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COMPARATIVE STUDIES ON LACTOBACILLI ISOLATED FROM THE MOUTH AND INTESTINE[1]

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From the Walter G. Zoller Memorial Dental Clinics and the Department of Bacteriology and Parasitology, University of Chicago, Chicago, Ill.

This paper is a report of a series of comparative studies made on lactobacilli isolated in this laboratory from the mouths and intestines of a small group of children and adolescents. The investigation is a part of a general program of study on the nature of the association of these organisms with dental caries. The primary purpose in undertaking this phase of the problem was to obtain information concerning the fate of oral lactobacilli during passage through the alimentary tract. Another, less immediate objective was the assemblage of data which could be used in certain projected phases of the general program, namely, study of the sources from which the oral lactobacilli become implanted in the mouth and examination of the relationship of oral lactobacillus incidence with food habits other than excessive carbohydrate indulgence.

The reports of earlier investigators who made comparisons between oral and intestinal lactobacilli contain variable and sometimes contradictory results. Some have presented evidence that these are different microorganisms (1–12), others that they are the same (13–26). During the course of previous studies in this laboratory (27–32) the characteristics of the oral lactobacilli had been found to extend over a considerable range. It had become apparent from these findings that, because of the rather marked differences among the oral lactobacilli, the results of any detailed comparison would depend to a large extent upon which oral strains were selected for study and perhaps also partly upon what intestinal strains were chosen. It seemed probable that these difficulties could be avoided in part, and perhaps entirely, by confining the study to lactobacilli isolated from the mouths and intestines of the same individuals. Hadley and Bunting (24) had made a comparative study of the morphological characteristics of cultures collected in this way but they did not report data on biochemical characteristics or immunological relationships.

Preliminary experiments on this subject were begun in 1938 when approximately 50 cultures of lactobacilli were collected from feces and saliva of 20 children who were available as outpatients of the dental clinics. These cultures, unfortunately, were isolated at irregular intervals because of the infrequency of the visits of some of the children to the clinics and because of the difficulty in getting cooperation from others. With this irregular schedule, by the time cultures had been accumulated in sufficient number to begin the study it had

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been found, in experiments with other oral lactobacilli isolated some time before, that many of the characteristics which were being used as a basis of comparison are apt to change within the first few months of laboratory cultivation (33). For that reason the study was stopped and the collection of another series of strains for this investigation was delayed until the characteristics of these changing oral strains could be analyzed. The isolation of cultures for the experiments reported in this paper was begun in the autumn of 1941.

METHODS

Sources of cultures: Twenty patients ranging in age from 7½ to 17 years, and hospitalized for various illnesses unrelated to oral conditions, were selected for study. The teeth of 18 patients were examined, and records were made of the degree and extent of dental caries involvement by members of the clinical staff. No dental treatment was given during the period of study except for fillings placed in teeth of 2 individuals. No dental records were available for 2 of the children. Each patient supplied approximately 10 cc. of saliva which was stimulated by chewing sterile paraffin and collected in sterile, wide-mouthed bottles. Stool specimens were obtained from each patient usually 24 to 48 hours after collection of the saliva samples. Specimens of saliva and feces were collected at intervals of 1 or 2 weeks until 3 to 5 paired samples had been obtained. Three of the patients were discharged from the hospital before the second or third specimens could be secured.

Methods of isolation: Specimens were placed in the refrigerator immediately after collection, were kept cold until prepared for study and were cultured as soon after collection as possible. Each saliva sample, thoroughly mixed by repeated, vigorous expulsion from a sterile pipette, was prepared in tenfold and hundredfold dilutions in sterile salt solution. Cultures were made in Jay's broth (34) and on acid tomato agar (35). Small masses of feces were emulsified in sterile salt solution, similar dilutions were prepared and inoculations were made in the same media. Cultures were incubated aerobically at 37° C. for 4 days. When no growth appeared on the plates the broth cultures were examined and if rod forms were present they were subcultured on acid tomato agar plates. Otherwise the broth cultures were discarded. Duplicate cultures of earlier stool specimens were incubated in anaerobic jars under partial CO_2 tension according to the method of Dack, Dragstedt and Heintz (36). Findings in these cultures were identical with those in cultures grown aerobically, however, and the anaerobic culture method was discarded in later work.

Routine culture procedure: Plate cultures were examined under a binocular colony microscope and representatives of each colony form present were described, stained and subcultured in Jay's broth. The first subculture from each was replated, reexamined for colony and cell morphology and further purified by single colony selection. Cultures were transferred routinely thereafter in Jay's broth. Morphological examinations were repeated after 4 to 6 months but consecutive subculture in Jay's broth was not broken by single colony isolation again.

Morphological examinations: All but the first of the colony examinations were made from cultures on brom-cresol-green, glucose, veal infusion agar (37), pH 7.2. Various forms making an almost continuous gradation from smooth, dome-shaped, glistening, semi-opaque colonies with sharp borders to rough, flat, nonglistening, translucent colonies with irregular or rhizoid margins were found in the series. They were classified as smooth, intermediate and rough, those which were on the borderline of 2 of these classifications being arbitrarily placed in the group which seemed most appropriate. In poured plate cultures the smooth strains produced colonies like the Y type of *L. acidophilus* described by Rettger and Horton (38) and the rough forms produced fuzzy colonies similar to the X type of these investigators. Gram stains were made of all the types of colonies found in each cultured specimen. Cell types were classified according to the groups described by Hadley, Bunting and Delves (19) although classification of both colony and cell morphology according to their criteria was not followed.

Fermentation reactions: Litmus milk and sorbitol and mannitol broths were inoculated from the first Jay's broth subculture and 10 other sugar broths were inoculated from either the first or second subculture. Cultures in milk and the sugar broths were incubated at 37° C. and were examined on the second, third, fourth, eighth and fifteenth days of incubation. Strains which did not ferment sorbitol and mannitol were retested at intervals of 3 or 4 weeks until they did ferment or until 6 or 8 successive negative reactions were obtained. Still negative strains were retested subsequently at less frequent intervals over a period of 15 months. All fermenting strains were also tested at these less frequent intervals.

Preparation of carbohydrate extracts: As soon as the reactions in litmus milk and in sorbitol and mannitol broth were known all the cultures from any 1 specimen, which appeared to be different from each other in morphology and fermentation reaction, were inoculated into 500 cc. quantities of Jay's broth. After incubation at 37° C. for 4 days these cultures were used for extraction of bacterial carbohydrate by a procedure previously described (28). When cultures changed in fermentation reactions in sorbitol and mannitol after the date of carbohydrate extraction, this procedure was repeated. Second extracts were prepared at a later date from many of the unchanging cultures also. The extracts were partially purified by subjecting them to 3 successive precipitations with 95% alcohol. The final alcohol precipitate was dissolved in 5 cc. of 0.85% salt solution and sufficient phenol was added to make a 0.2% solution of the preservative. Previous experience had indicated that the yield of carbohydrate is usually between 1 and 2 milligrams per liter of culture. Hence it may be assumed that the amount contained in the 5 cc. amounts prepared here, obtained from 500 cc. of culture, was about 0.5 to 1.0 mgm. These solutions probably, therefore, represent approximately 1:500 or 1:1000 dilutions of the carbohydrate substance.

Immunological tests: Antisera for 10 type strains of oral lactobacilli, prepared by the injection of rabbits with heat killed vaccines, were used for precipitation tests with the bacterial carbohydrate extracts. Undiluted serum in 0.25 cc. amounts was mixed in agglutination tubes with equal amounts of the diluted carbohydrate solutions (the original solution and 1:5 and 1:50 dilutions of it) making final estimated dilutions of 1:1000, 1:5000 and 1:50000 to 1:2000, 1:10000 and 1:100000 of the carbohydrate substance. Tubes were incubated at 37° C. for 2 hours, stored in the refrigerator overnight and read the next day

EXPERIMENTAL RESULTS

Studies were made on 68 saliva samples obtained from 20 children. All but 2 of these samples were paired with stool specimens. The pertinent data regarding caries findings, dates of sampling of saliva and feces, presence or absence of lactobacilli and morphological characteristics of the strains isolated are presented in Table I.

Salivary lactobacilli and caries activity

As previously indicated all the subjects used in the study were patients hospitalized for various illnesses unrelated to dental caries. They were selected at random and preliminary inquiry into the oral conditions was purposely avoided. All but 2 of them were under observation or treatment in the Zoller Dental Clinics. The dental records were not consulted by the authors, however, until the studies recorded in this paper were completed. Dental histories were not obtained from 2 patients. One, No. 1, was almost free of lactobacilli, 2 of the saliva specimens yielding negative cultural results and only 2 colonies of lactobacilli developing in the cultures from the third saliva sample. The other of these 2 patients, No. 12, provided 5 saliva samples, of which the first was negative and the others contained lactobacilli in small numbers (150, 1800, 120 and

TABLE I

Lactobacillus isolations from mouths and intestines of 20 children with varying caries experience

PATIENT	SEX AND AGE	AMOUNT OF CARIES PRESENT	EXAMINATION DATES		MORPHOLOGICAL TYPES OF LACTOBACILLI FOUND*	
			Oral	Intestinal	Oral	Intestinal
1	Male 8	No dental examination	11/17/41 12/ 1/41 12/15/41	11/18/41 12/ 2/41 12/15/41	none sII none	none none none
2	Male 11	8 cavities in 4 deciduous and 4 permanent teeth	11/17/41 12/ 5/41 12/15/41	11/19/41 12/ 6/41 12/16/41	sI sI; iIII, iR sI	sII sII none
3	Female 12½	No caries	11/17/41		none	
4	Male 12	14 cavities	11/17/41 12/ 1/41 12/15/41 12/29/41	11/19/41 12/ 2/41 12/17/41 1/ 2/42	sII sII none iII	none none none none
5	Male 13½	7 proximal lesions. 15 surfaces previously filled and 3 permanent molars extracted	11/17/41 12/ 1/41 12/15/41 12/29/41	11/18/41 12/ 2/41 12/17/41 12/29/41	sI (2); II iIII; rR sII; iII; iR sII; iIII; rR	iII iIII iII sII
6	Male 13	27 carious surfaces. 17 proximal and 10 occlusal lesions	11/17/41 12/ 1/41 12/16/41 12/29/41	11/18/41 12/ 2/41 12/17/41 12/29/41	sI(2); iIII sII; rR sI sII	sI sII sI; rR sI
7	Male 7½	No caries, 4 fillings	11/17/41 12/ 1/41 12/15/41	11/18/41 12/ 2/41 12/17/41	none sI none	none none none
8	Male 10	No caries. A few deciduous teeth missing. Occlusal fillings in 2 permanent molars	11/17/41 12/ 1/41 12/15/41	11/18/41 12/ 2/41 12/17/41	none sII none	none none none
9	Female 11	No caries. 3 deciduous teeth missing. 1 permanent 1st molar missing. 4 fillings, last made on 11/13/41	11/17/41 12/ 1/41 12/15/41	11/18/41 12/ 2/41 12/15/41	none sII none	none none none
10	Female 11	No caries. 3 fillings, placed on 10/10/41 and 10/17/41	11/17/41 12/ 1/41 12/17/41	11/18/41 12/ 2/41 12/17/41	none sII none	none none none

* Colony form (s—smooth, i—intermediate, r—rough) and cell morphology (groups I, II, III and Rough of Hadley, Bunting and Delves (19)). The numbers in parentheses refer to second strains of slightly different morphology.

TABLE I—*Continued*

PATIENT	SEX AND AGE	AMOUNT OF CARIES PRESENT	EXAMINATION DATES		MORPHOLOGICAL TYPES OF LACTOBACILLI FOUND*	
			Oral†	Intestinal	Oral	Intestinal
11	Female 13	Fillings in 4 permanent teeth, placed 8/1/40. 2 pit cavities found on 1/3/42. 6 new lesions found on 6/5/42.	11/24/41 12/ 8/41 12/15/41 1/ 5/42	11/25/41 12/10/41 12/17/41 1/ 6/42	sII sII sII none	sII none rR none
12	Female 9	No dental examination	11/24/41 12/ 8/41 12/15/41 12/22/41 1/ 5/42	11/25/41 12/10/41 12/17/41 1/ 6/42	none rR iII iII iII	none rR iII iII
13	Female 9	2 cavities in deciduous teeth	11/24/41 12/ 8/41 12/15/41 1/ 5/42	11/25/41 12/10/41 12/17/41 1/ 6/42	sI sII sI sI	none sII none none
14	Female 17	5 large cavities	11/24/41 12/ 8/41 12/15/41 1/ 5/42	11/25/41 12/10/41 12/17/41 1/ 6/42	sI iR sI sII	iIII iR sI; iR none
15	Female 8	9 carious lesions on 11/24/41; 4 filled on 12/9/41, 2 on 12/31/41 and 3 on 1/2/42. Several new ones when seen on 10/13/42	11/24/41 12/ 8/41 12/15/41 1/ 5/42	11/25/41 12/10/41 12/17/41 1/ 6/42	sI sII sI sI	sI sII; iIII iII sI
16	Male 10½	8 carious permanent teeth	11/24/41 12/ 8/41 12/15/41 12/22/41 1/ 5/42	11/25/41 12/ 8/41 12/16/41 12/23/41 1/ 6/42	sII; iII sI, sII; iIII sI; iR sI; iR sI	iII, iIII sII sI, sII sI, sII sI
17	Male 12	7 carious permanent teeth	11/24/41	11/25/41	sII	none
18	Male 11	14 carious permanent teeth on 11/24/41. 5 filled before 12/8/41. 7 before 12/15/41, 9 before 1/5/42. (Larger lesions eliminated first.)	11/24/41 12/ 8/41 12/15/41 1/ 5/42	11/25/41 12/10/41 12/17/41 1/ 6/42	iR sII sI none	sI sII none none

TABLE I—*Concluded*

PATIENT	SEX AND AGE	AMOUNT OF CARIES PRESENT	EXAMINATION DATES		MORPHOLOGICAL TYPES OF LACTOBACILLI FOUND*	
			Oral	Intestinal	Intestinal	Oral
19	Female 17	No caries. 1 occlusal pit filled 10/24/41. First new lesions seen 8/27/42 (Occlusal pits)	11/24/41 12/ 8/41	11/25/41 12/10/41	none sII	none none
20	Female 12	Mouth not examined since 1/10/40 at which time there were 3 small proximal lesions and 4 fillings	11/24/41 12/ 8/41 12/15/41 1/ 5/42	11/25/41 12/10/41 12/17/41 1/ 6/42	sII sII sII sII	none sII none sII

350 per cubic centimeter). Two, Patients 3 and 17, were released from the hospital before more than 1 sample was collected.

Five of the remaining 16 patients had no discernible caries at the time of this study. Cultures were made from 14 saliva specimens from these 5 patients and lactobacilli were found only 5 times, once from each patient. All of the counts were low, one being 80 per cc., one 30 per cc. and three 20 per cc. each. There was definite caries activity in the other 11 subjects. From them there were 44 samples of saliva, not less than 3 specimens from any 1 child, and only 3 specimens were negative for lactobacilli.

The numbers of lactobacillus colonies developing in cultures from successive saliva samples were fairly uniform in some cases and were variable in others. Such results have been common in our experience and have been frequently reported by others. The maximum counts for each case ranged from 6000 to 150,000. Patients 2, 4, 5 and 11 had counts of 12,000 or below, Patients 6, 13, 15 and 18 were between 20,000 and 40,000 and Patients 14, 16 and 20 had more than 80,000 lactobacilli per cc. of saliva.

The lactobacillus count of Patient 18 was 40,000 per cc. on the first examination at which time there were 14 carious lesions. Before the next sample was collected 5 of the largest lesions had been removed by appropriate dental treatment. The count dropped to 50 in the second examination and to 20 in the third examination after 2 more lesions had been eliminated. On the fourth examination when only 5 small cavities remained the saliva sample was negative. Patient 15, however, had no reduction in count accompanying removal of lesions. Her last sample, collected when all the recognized cavities had been treated, had the highest count. It should be stated, however, that since radiographic examination with critical bite-wing films was not attempted in this series, it is possible that early lesions could have been present without being detected. Several new lesions were discovered when she was examined again about 10 months later. Whether or not this indicates that there was caries activity at the time the last sample was collected is conjectural.

Although this is a small series the results are in general agreement with the

findings from more comprehensive studies, conducted in this laboratory and elsewhere, on the use of the salivary lactobacillus count as an indicator of dental caries activity. In general, high counts have been found to be associated with extensive decay and low counts with minimal caries activity. There is, however, a rather broad individual variation in the numbers of organisms found, at any level of caries involvement (29, 30), with the result that the correlation of lactobacillus count and extent of carious damage is not a close one. In the present series, for example, the highest counts are not always associated with the greatest amount of decay and reduction of the number of lesions was not always accompanied by reduction in the lactobacillus count.

Occurrence of lactobacilli in the intestine in relation to salivary lactobacillus count

Cultures of lactobacilli were obtained from 52 (76%) of the 68 samples of saliva. Neither the broth nor agar media inoculated with the other 16 samples contained these organisms. No lactobacilli were found in 34 stool specimens. They were isolated, however, from the remaining 32 specimens and when found were usually present in large numbers.

The 11 carious patients provided 44 stool specimens of which 29 were found to contain lactobacilli. Fifteen stool specimens were from the 4 patients (Nos. 2, 4, 5 and 11) whose maximal salivary counts were below 12,000 and 7 of them failed to yield lactobacilli in cultures. The four (Nos. 6, 13, 15 and 18) whose highest salivary counts ranged between 12,000 and 40,000 provided 16 stool specimens and 11, or nearly three-fourths of them contained lactobacilli. Thirteen stool specimens were cultured from the 3 patients (Nos. 13, 16 and 20) whose maximal salivary counts were above 80,000 per cubic centimeter and lactobacilli were found in all but 3 of the specimens.

In the whole series of 19 cases which provided 1 or more of the 66 stool specimens studied, no fecal sample yielded lactobacilli in culture when the corresponding saliva specimen was negative. Fifteen of the 37 negative stool cultures were from specimens paired with negative saliva samples. Of the stool specimens paired with 19 saliva samples which had counts below 1000 per cc., only 5, or approximately one-fifth, were positive for lactobacilli. Among the 32 stool specimens paired with saliva samples having counts above 1000 per cc., 24 or three-fourths yielded lactobacilli in culture.

These findings are paralleled to some extent by the results reported by Hadley and Bunting (24) who recorded 1 caries-free individual as having 4 successive negative saliva cultures with 4 corresponding fecal samples being also negative.

Morphological characteristics

It may be seen from the data recorded in Table I that lactobacilli of the same morphological groups were found in the feces and in the saliva. In 23 of the 32 positive intestinal cultures the cell and colony morphology were the same as shown by strains obtained from the saliva at the same culture period. The occurrence of morphologically identical strains in the mouth and intestine was noted also by Hadley and Bunting (24). Smooth strains predominated in both the oral and intestinal groups, 48 of the 68 oral strains and 23 of the 38 intestinal

strains having smooth colony form. This also is confirmatory of the results of Hadley and Bunting who found smooth forms predominant in feces as well as in saliva. Approximately half the strains in each group, 31 oral and 19 intestinal, had cell morphology corresponding to Group II of Hadley, Bunting, and Delves (19). More of these had smooth colony form than the flattened, semi-rough form designated as "intermediate." Small numbers of long celled, Group III strains, and of swollen, irregular, pleomorphic or rough strains were isolated from both saliva and feces.

Since the primary purpose of the study was to compare lactobacilli isolated from the intestines with those obtained from the mouths of the same children only those strains that were isolated from patients yielding cultures from both saliva and stools were subjected to further investigation. Ten oral lactobacillus strains obtained from Patients 1, 4, 7, 8, 9, 10, 17 and 19 were eliminated and, since there were no lactobacilli obtained from Patient 3, the remainder of the study concerns only the cultures obtained from 11 children.

In studying the final collection of 58 oral and 38 intestinal cultures 2 general procedures were followed. The data collected on all the cultures in each group were combined first for comparison of the range of characteristics, and then studied separately for evidence of identity or lack of identity of oral and intestinal organisms isolated from the same individuals. The characteristics studied were cell and colony morphology, fermentation reactions and immunological types as determined by carbohydrate precipitation tests. It was well known that the oral lactobacilli are unstable in morphology, particularly in the case of freshly isolated cultures. It was known also that many cultures change in fermentation reactions after a variable period of laboratory cultivation (20, 21, 27, 33, 39) and that the immunological specificity may also change (33). For these reasons all the observations and tests were repeated 1 or more times on all strains.

The distribution of morphological groups among the oral and intestinal strains, at the time of isolation and after a period of laboratory cultivation, is shown in Table II. When examined shortly after isolation the intestinal strains included representatives of all the morphological forms found in the saliva cultures with the exception of the intermediate type of colony with "rough" cell morphology and only 3 of these were found in the oral group. The distribution of groups was different, however, there being proportionately more intermediate and rough forms among the intestinal strains than among the oral strains. This finding is in keeping with the observations reported by Rettger and associates (6–8, 11) although the distinction is not nearly as marked as they found. Perhaps the most marked difference between the intestinal and oral strains was in the occurrence of Group II cells. Counting both those with smooth colony form and those with intermediate colonies, 63% of the intestinal strains were in Group II as compared with 38% of the oral strains. Hadley and Bunting (24) found their Group II lactobacilli somewhat more common in the feces than in the saliva, their figures being 36% of the fecal strains as compared with 26% of the oral strains in the case of caries-susceptible individuals and 15% and 4% respectively for the caries-free individuals.

During the course of laboratory cultivation there was a shift of both oral and intestinal strains toward smooth colony form and Group I cell morphology and this shift was more marked in the cultures of fecal origin than among those from the mouth. The result of these shifts was that after 4 to 6 months of cultivation in Jay's broth the distribution of the 2 collections was almost identical. A summary of the morphological findings is given in Table II. Smooth Group I had increased in the oral collection from 36% of the total at the time of isolation to 62% after a few months of cultivation and in the intestinal collection from 16% to 61% of the total. The numbers of strains remaining in almost all the other classifications were decreased and there were no rough cultures left in

TABLE II

Distribution by morphological groups of 96 strains of oral and intestinal lactobacilli, in original cultures and after continued growth in Jay's broth

COLONY FORM*	CELL TYPE**	AT TIME OF ISOLATION				AFTER CULTIVATION FOR 4 TO 6 MONTHS			
		Numbers		Percentages		Numbers		Percentages	
		Oral	Intestinal	Oral	Intestinal	Oral	Intestinal	Oral	Intestinal
Smooth	I	21	6	36	16	36	23	62	61
	II	18	14	31	37	13	9	22	24
	Total	39	20	67	53	49	32	84	85
Intermediate	II	4	10	7	26	5	4	9	10
	III	7	3	12	8	4	2	7	5
	R	3	0	5	0	0	0	0	0
	Total	14	13	24	34	9	6	16	15
Rough	R	5	5	9	13	0	0	0	0
Totals.....		58	38	100	100	58	38	100	100

* Smooth: raised, dome, glistening. Intermediate: flat, translucent, granular. Rough: flat, uneven, feathery; buried colonies like the "X" type described by Rettger and Horton (38).

** I, II and III refer to morphological groups of Hadley, Bunting and Delves (19), "R" indicates pleomorphic, threadlike or swollen forms, typical of cells from rough colonies.

either collection after 6 months. It is apparent that growth in the laboratory under the conditions supplied was favorable to dissociation into the smooth form. The direction of change was not invariable, however, since a few cultures in both collections changed from smooth to intermediate colony form and from Groups I or II to Groups II and III.

The eventually almost identical morphology of the strains in the 2 collections would indicate that, unless they should prove to be different in biochemical or other properties, the oral and intestinal strains included the same species. Since there were considerable differences in the percentage distribution of the various morphological groups at the time of isolation it may be assumed, if the organisms should prove to be members of the same species, that either the oral habitat favors development of the smooth form or the intestinal habitat tends to impose

dissociation away from this form. Inasmuch as part of the basis for the distinction between oral and intestinal lactobacilli, which has been made by several investigators (3, 5–11), has been such observed differences in morphology, further experimental test of this assumption would probably be of some value. Such tests, however, were not a part of this study.

Fermentation reactions

As indicated above, fermentation tests were made as soon as possible after isolation of the cultures and were repeated 1 or more times thereafter at short

TABLE III

Comparison of fermentative capacities of oral and intestinal lactobacilli at time of isolation and after continued growth in Jay's broth

SUBSTANCES TESTED	NUMBER OF STRAINS:								PERCENTAGE POSITIVE			
	Oral (58 strains)				Intestinal (38 strains)				First tests		Later tests	
	Positive in first test	Losing reactivity	Gaining reactivity	Positive in later test	Positive in first test	Losing reactivity	Gaining reactivity	Positive in later test	Oral	Intestinal	Oral	Intestinal
Litmus milk												
Acid	58	0	0	58	38	0	0	38	100	100	100	100
Coagulation	48	4	8	52	34	1	2	35	83	90	90	92
Reduction	43	4	12	51	31	1	5	35	74	82	88	92
Glucose	58	0	0	58	38	0	0	38	100	100	100	100
Levulose	56	0	2	58	36	0	2	38	97	95	100	100
Arabinose	43	0	15	58	31	0	6	37	74	82	100	97
Xylose	52	1	6	57	37	0	1	38	90	97	98	100
Maltose	56	1	2	57	36	0	1	37	97	95	98	97
Sucrose	54	1	3	56	36	0	1	37	93	95	97	97
Galactose	55	3	3	55	33	0	4	37	95	87	95	97
Mannitol	39	4	15	50	30	1	7	36	67	79	86	95
Sorbitol	34	4	15	45	26	1	7	32	59	68	78	84
Raffinose	50	25	5	30	28	10	5	23	86	74	52	61
Inulin	13	2	14	25	10	3	8	15	22	26	43	39
Dextrin	12	11	0	1	4	4	1	1	21	11	2	3

or long intervals depending upon the results of the first tests. In Table III the reactions of 58 oral strains and 38 intestinal strains in litmus milk and 12 carbohydrate broths are summarized. All strains produced acid in milk and all fermented glucose in both early and late tests. Acid production in milk although characteristic of all strains was, as would be expected, slight in the case of strains that failed to coagulate it. Ten oral strains originally failed to coagulate milk and 4 that coagulated milk in first tests later failed to do so. These 10 and 5 others failed to reduce litmus. The 4 strains which lost the ability to coagulate milk also lost the power to reduce litmus. Eight strains not coagulating milk in early tests, and 12 which failed to reduce litmus, later gained these properties.

The percentages of intestinal strains having similar early reactions and like changes in later tests were not significantly different.

The carbohydrates tested included arabinose, dextrin, galactose, inulin, levulose, maltose, mannitol, raffinose, sorbitol, sucrose and xylose as well as glucose. They are arranged in Table III in decreasing order according to the number of strains fermenting them in later tests. All strains which fermented the carbohydrates produced sufficient acid to give the full color change of the indicator used, brom-cresol-purple, but none formed gas. The major changes were in reaction in arabinose, sorbitol, mannitol and inulin, in which many strains which were negative at first were positive in later tests and in raffinose and dextrin which several strains lost the power to ferment.

The oral and intestinal strains in the first tests were nearly identical in the ability to produce acid in milk, glucose, levulose, maltose and sucrose but differed by 4% to 12% in proportions positive in the other carbohydrates. In the later tests, however, the percentages of the 2 groups with positive reactions were almost identical in all but 3 test substances Both groups gained in the proportions fermenting mannitol and sorbitol and both lost in fermentation of raffinose but percentage differences between the groups remained about the same. Taking the reactions of the 2 groups as a whole they were remarkably similar, particularly after a period of growth in laboratory media. As with the morphological characteristics summarized in Table II this summary of the fermentation reactions gives no indication that the oral and intestinal organisms studied in this series are fundamentally different from each other.

Comparison of fermentation patterns of the oral and intestinal strains is made in Table IV. Since most of the strains in both groups produced acid in milk, levulose, galactose, xylose, maltose and sucrose and since only 2 strains fermented dextrin in the later tests, this comparison has been made on the basis of coagulation and reduction in litmus milk and acid production in the other 5 carbohydrates.

In the first tests, made shortly after isolation of the cultures, there were 42 different combinations of fermentation reactions. More than half of these combinations represented the reactions of single strains. When all combinations shown by not more than 2 strains were eliminated, 50% of the oral strains and 63% of the intestinal cultures fell into the remaining 8 fermentation groups. All of them fermented levulose, galactose, xylose, maltose and sucrose and all were negative in dextrin but differed in action in arabinose, raffinose, mannitol, sorbitol, inulin or litmus milk. When tested again after 3 to 6 months, between 85% and 90% of the strains in both collections fell into these 8 fermentation groups. Only 12 strains remained in the heterogeneous group and a few of these, as found by retests about a year later, changed subsequently to one of the 8 patterns shown in Table IV. Tests were not repeated on the whole group, however, and are therefore not shown in the table.

Although the percentages of the oral and intestinal strains falling into the several groups differ somewhat they are remarkably close considering the amount of changes in reaction which occurred after the cultures had been grown for

some time in laboratory media. It was noted in an earlier section of this paper that the fermentation reactions of freshly isolated cultures of oral lactobacilli have been known for some time to be unstable. The results obtained here with the cultures isolated from the fecal specimens indicate that this is characteristic also of lactobacilli from that source. It was observed in these experiments, as well as previously, when some of the cultures were tested several times, that changes in reaction appear early with some cultures and much later with others and that the changes of a given strain in ability to ferment different sugars do not all take place at the same time.

TABLE IV

Chief fermentation groups of oral and intestinal lactobacilli, in tests made at time of isolation and after continued growth in Jay's broth

CLASSIFICATION AT TIME OF ISOLATION		FERMENTATION REACTION						CLASSIFICATION AFTER CULTIVATION FOR 3 TO 6 MONTHS	
Numbers and approximate percentages		Litmus milk*	Arabinose	Raffinose	Mannitol	Sorbitol	Inulin	Numbers and approximate percentages	
Oral (58 strains)	Intestinal (38 strains)							Oral (58 strains)	Intestinal (38 strains)
8 (14%)	4 (11%)	+	+	+	+	+	+	12 (21%)	9 (24%)
11 (19%)	8 (21%)	+	+	+	+	+	−	10 (17%)	10 (26%)
2 (3%)	3 (8%)	+	+	+	+	−	−	3 (5%)	2 (5%)
2 (3%)	2 (5%)	+	+	+	−	−	−	1 (2%)	0
0	1 (3%)	+	+	−	+	+	+	11 (19%)	6 (16%)
2 (3%)	5 (13%)	+	+	−	+	+	−	10 (17%)	6 (16%)
2 (3%)	1 (3%)	+	−	+	+	−	−	0	1 (3%)
2 (3%)	0	−	+	+	−	−	−	3 (5%)	0
29 (50%)	24 (63%)	Totals**						50 (86%)	34 (89%)
29 (50%)	14 (37%)	34 other combinations†						8 (14%)	4 (11%)

* Coagulation and reduction; all produced some acid in milk. In sugar broths + indicates acid production; −, no acid.

** All positive in levulose, galactose, xylose, maltose and sucrose. All negative in dextrin.

† Including reactions in levulose, galactose, xylose, maltose, sucrose and dextrin; 25 of these combinations exhibited by oral strains and 16 by intestinal strains.

Immunological type relationships

In previous studies on oral lactobacilli (28, 32) it had been found that the cellular carbohydrate substance, a water soluble, alcohol insoluble, acid extract of the cells, reacted type specifically in antibacterial serum and that most of the cultures that were isolated from dental plaques and saliva samples of caries patients could be typed by means of this reaction. At the time this study was begun this method had not been used on lactobacillus cultures from other sources. It was assumed, however, that if the cultures isolated from the intestine were members of the same species as the oral lactobacilli the same immunological types should be represented.

Extracts were prepared from selected cultures of both oral and intestinal origin. All the strains collected from each individual were grouped separately and, insofar as possible, extracts were prepared from all those that appeared to be different from each other in morphology or fermentation reactions. Since the preparation of the carbohydrate extracts is time consuming it was neither desirable to make tests on all the cultures in the 2 collections nor feasible to prepare many extracts at one time. When cultures changed in reaction in sorbitol and mannitol subsequent to the extraction of carbohydrates, and such changes were found in 20 of the strains studied, second extracts were prepared and tested. Other strains, in equivalent number, not changing in fermentation reaction were reextracted and retested as controls. In a few instances when strains fluctuated in fermentation reactions three or four different carbohydrate extracts were prepared.

The results with all the carbohydrate extracts prepared from cultures obtained from one patient, No. 5, are shown in Table V together with the results of fermentation tests of these cultures in sorbitol and mannitol. Similar tests were made with cultures from all the 11 patients studied and the results are presented in the summary tables which follow. The results with cultures from this patient are given as detailed examples. Since the carbohydrates were not highly purified before testing it is likely that the minor reactions, shown in Table V as $\pm$ reactions of extracts from 5 cultures, were due to the presence of traces of nucleoprotein or other antigen common to the species, i.e., it is probable that these incomplete reactions do not indicate cross relationships between the different types. If the incomplete reactions are ignored it will be seen that 5 of the cultures reacted specifically with single type sera. Five strains reacted apparently specifically, however, with 2 sera and 1 with 3.

More than 1 carbohydrate extract was made from 8 of the 11 cultures listed in Table V. If the minor or trace reactions are again ignored, there is no significant difference between the earlier and later extracts of 4 of the 8 in their reactions in antiserum. A fifth strain, Zs5k, which was reactive with 2 antisera at the time of the first extraction, yielded a second extract which reacted with a third antiserum. All of these 5 strains fermented sorbitol and mannitol at the time of carbohydrate extraction. Three strains, Zs5g, Zs5i and Zi5i, gained type-specificity with acquisition of the ability to ferment sorbitol and mannitol, being readily typed when able to ferment and reacting in none of the type sera when not able to ferment. One of them, Zs5i, precipitated in 16A serum at a time when the culture fermented mannitol, lost typability when it lost the capacity to ferment mannitol and gained typability, this time in 2 other antisera, 26A and 96A, when it acquired the ability to ferment both sorbitol and mannitol. These changes are analogous to the changes in specificity previously reported from this laboratory (33).

Precipitation tests were performed with 1 or more carbohydrate extracts from each of the 37 strains isolated from saliva and 30 strains obtained from stool specimens. At least 1 oral and 1 intestinal strain from each of the 11 patients was tested. The results are summarized in Table VI. All but 2 of the oral

TABLE V

Precipitation reactions of bacterial carbohydrates and their relationship to fermentation reactions

Tests with cultures from a single patient

CULTURES TESTED			DATES OF CARBOHYDRATE EXTRACTION	FERMENTATION REACTIONS* AT OR NEAR TIME OF CARBOHYDRATE EXTRACTION		CARBOHYDRATE PRECIPITATION REACTIONS** IN TYPE-SPECIFIC ANTISERA					
Source	Strain numbers	Dates of isolation		Sorbitol	Mannitol	26A	96A	45A	16A	19A	9A
Mouth	Zs5a	11/17/41	2/ 6/42	+	+	+	+	−	−	±	±
	Zs5d	12/ 1/41	2/ 6/42	+	−	−	−	−	−	−	+
			5/ 8/42	+, −	+, −	−	−	−	−	−	+
			10/15/42	+	+	−	−	−	−	−	+
	Zs5f	12/17/41	9/ 9/42	+	+	+	+	−	−	±	±
	Zs5g	12/17/41	1/12/42	−	−	−	−	−	−	−	−
			5/ 4/42	+	+	+	+	−	−	−	−
	Zs5h	12/17/41	1/ 7/42	+	+	−	−	−	−	+	−
	Zs5i	12/19/41	2/14/42	−	+	−	−	−	+	−	−
			5/ 8/42	−	−	−	−	−	−	−	−
			9/ 4/42	−	−, +	−	−	−	−	−	−
			3/ 4/43	+	+	+	+	−	−	−	
	Zs5k	12/29/41	2/ 6/42	+	+	+	−	+	−	−	−
			6/12/42	+	+	+	−	+	−	+	−
Intestine	Zi5a	11/18/41	9/ 9/42	−	+	+	−	±	−	−	±
			1/ 7/43	+	+	+	±	±	±	±	±
			3/ 9/43	+	+	+	±	±	±	−	±
	Zi5d	12/ 2/41	2/14/42	+	+	+	−	−	−	−	−
			1/ 7/43	+	+	+	−	−	−	−	−
	Zi5f	12/15/41	1/10/42	+	+	+	+	−	−	±	±
			2/27/43	+	+	+	+	−	−	−	−
	Zi5i	12/29/41	2/14/42	−	−	−	−	−	−	−	−
			6/12/42	−	−		−		−	−	−
			9/ 4/42	−, +	−, +	−	−	−	−	−	−
			2/ 4/43	+	+	+	±	±	−	−	−

* Fermentation reactions: + = acid formed in 3 to 7 days; − = no acid formed in 14 days. Double entries are made when tests were made before and after, but not on, the dates indicated and if the reactions changed during the interval.

** Reactions in serum: + = cohesive, scale-like precipitate, difficult to disperse, unclouded supernatant; ± = cloudy supernatant; sediment, if any, loose and easily dispersed; − = no visible reaction or only faint clouding in lower dilutions.

strains and 3 of the fecal strains reacted in antiserum. Almost half the carbohydrate extracts precipitated with antisera 26A and 96A and the proportions of the oral and intestinal collections so reacting were not significantly different. It will be noted that comparatively few of the strains reacted in 26A antiserum alone or in 96A serum alone. A few strains which reacted in 26A also reacted in 45A antiserum. None of the extracts precipitated, however, in both 45A and 96A antisera. Whether these reactions indicate some overlapping of carbohydrate antigens between strains 26A, 45A and 96A, from which the antisera were prepared, or whether they result only from antigenic complexity of the carbohydrates of the test strains cannot be determined from information available at present. The results of previous tests for cross reactions between the original type-specific carbohydrates and their antisera had shown some relationship between 26A and 96A, but none between 26A and 45A or 26A and 19A such as are indicated by some of the reactions listed in Table V and indicated in Table VI. For that reason it is assumed that the cross reactions exhibited by these cultures probably result from the presence of more than one type-specific

TABLE VI

Immunological relationships of lactobacilli of oral and intestinal origin, as determined by carbohydrate precipitation tests

SOURCE OF CULTURES	NUMBER TESTED	NUMBER REACTING IN TYPE-SPECIFIC ANTISERA						
		26A	96A	45A	26A and 96A	26A and 45A	Others*	Total reacting
Oral	37	2	2	9	14	3	5	35
Intestinal	30	2	3	3	15	2	2	27

* Four other antisera (4A, 9A, 16A and 19A).

component in the extracts tested although it is possible that the reactions indicate an inter-relationship between types which was not apparent in earlier tests.

As was the case with the morphological groups and with the fermentation groups there is a similar distribution of immunological types within the 2 collections of cultures. The greatest difference found is in the number of strains reacting with 45A antiserum. We believe the results of these tests taken together with the morphological observations and the results of the fermentation tests justify the conclusion that the cultures isolated from the fecal specimens are members of the same species as those isolated from the saliva.

Direct comparison of oral and intestinal cultures

It was assumed when the study was begun that if the intestine contains lactobacillus species not represented in the oral flora, and that if swallowed oral lactobacilli pass through the alimentary tract without becoming implanted or without extensive multiplication in the intestine, it might be difficult to find the same oral lactobacilli in the stools as would be isolated from the saliva in paired samples

collected on the same day. This assumption was predicated on the further assumption that the lactobacilli predominant in the mouth on one day could disappear or be submerged by other strains on another day, i.e., that some of the patients might have a fluctuating oral flora. In order that the data obtained should provide the proper information if these possibilities proved to be fact, the stools were obtained for culturing 24 to 48 hours after the saliva samples were collected.

The second of the 2 assumptions made above seems logical when it is remembered that a good many different foods contain lactobacilli and when account is taken of the possibility that the oral flora may be determined by or may be quickly modified by the various strains introduced from such sources. Experience in this laboratory had indicated that considerable fluctuation in strains may be expected from some persons. It would have been useful in this regard to have a series of saliva samples and stool specimens from each individual, collected daily or, if possible, at more frequent intervals, but this was not feasible because of the amount of work involved in final identification of all the cultures collected. It was planned, therefore, to collect paired samples at intervals of about one week over a period of a month or so. If certain strains are fixed inhabitants of the mouth or intestine it was believed that this fact should become apparent from this minimal amount of reinvestigation.

As indicated earlier in this paper, it was known that many of the oral lactobacilli change in morphology, fermentation reactions and immunological relationships during the course of laboratory cultivation and the cultures obtained from both saliva and stool specimens were studied with this possibility in mind. They were examined as soon as possible after isolation, were watched for changes during the succeeding months and were restudied after changes had occurred. Analysis of all these variations and discussion of the factors involved, the significance of the changes, etc., will be left for another paper on this subject. Some of the changes have had to be taken into account in this paper, however, in order to make clear the relationships discussed here. For example, cultures from the mouth and intestine of a given individual which appeared to be alike in several characteristics at the time of isolation proved in some instances to be immunologically different at a later time. Also occasionally the converse was true, namely, that cultures of oral and intestinal origin, different in 1 or more respects when first studied, later proved to be identical in all the properties investigated. Many of the cultures did not change, however, or if changes appeared they were in minor characteristics only. Few of the cultures that typed shortly after isolation underwent any subsequent change either in immunological relationships or other characteristics. Most of those which yielded carbohydrate extracts that were not reactive in the available antisera did undergo changes before it became possible to type them.

Including both the cultures that changed in 1 or more characteristics and those which did not undergo such change, there are at least 12 pairs of oral and intestinal cultures that eventually proved to be identical or nearly so, by all the tests that were made. The chief characteristics of 12 such pairs after they

became sufficiently stabilized to be typed immunologically are presented in Table VII.

The double entries in the columns on morphology and fermentation record observations made before and after the carbohydrate extracts were prepared, tests not having been made at the time of extraction and change having occurred during this interval. The morphological changes, which appeared in 3 cultures, were not marked and in each case one or the other of the forms was identical with that of the corresponding culture from the other source. The changing fermentation reactions in 6 of the 11 instances were identical for the paried cultures. The changes were reversed in 1 case, however, and in 4 cases they occurred with only 1 of a pair of strains.

In previous investigations (32, 33) it had been found that fermentation reactions in sorbitol and mannitol are in some way related to the immunological typing of the oral lactobacilli. No such relationship has been found in the case of raffinose and inulin. The only changes that occurred in sorbitol and mannitol fermentation are those recorded for Zs16g and Zi16b, 2 strains which typed in 45A. It is interesting that the only other strains which typed alone in 45A failed to produce acid in sorbitol and mannitol.

The multiple entries in the column of serum precipitation reactions represent positive reactions of the same carbohydrate extract in more than 1 antiserum. Although the occurrence of such complex antigenic relationship is interesting and the explanation of these reactions is perhaps of considerable importance, it is sufficient to note for the purposes of this study that the paired strains are identical, with the single exception of Zi18b which had a secondary reaction in serum 45A not shown by its paired culture.

Except for the differences noted in the preceding paragraphs the 12 pairs of cultures were apparently identical. Since both members of each pair of cultures were obtained from the same patient it seems probable that they came originally from the same strain, that is, that each of the oral cultures arose from previously established intestinal strains or that the intestinal cultures represent swallowed oral lactobacilli. Although either origin is possible it seems likely that the latter one actually fits the case. Assuming that this is true it is of some importance to study the relationship in time of isolation of the cultures from the original specimens. Both cultures of 1 pair were isolated on the same day. The intestinal cultures in 7 pairs were isolated from fecal specimens collected 24 to 48 hours after the corresponding saliva samples were obtained. Among the other 4 pairs the intestinal cultures were isolated 4 weeks after the oral cultures in 2 instances and 5 days and 6 weeks before in 2 instances.

These findings indicate that if the mouth is the origin of the intestinal lactobacilli some of the strains at least must have been established in the oral flora for several weeks before the date of isolation from the saliva and some must have persisted in the saliva or have become implanted in the intestine for at least a few weeks before the date of recovery from the stools. Either or both of these conditions could have been true without becoming evident in the study. One or more strains differing in fermentation reaction and immunological

TABLE VII

Comparison of lactobacilli isolated from mouth and intestine, twelve pairs of cultures isolated from eight children with dental caries

CULTURE NUMBERS	SOURCE	DATE OF ISOLATION	CHARACTERISTICS AT TIME OF IMMUNOLOGICAL TYPING					
			Morphology[a]	Fermentation[b] reactions				Positive serum-precipitation reactions[c] in:
				Raffinose	Sorbitol	Mannitol	Inulin	
Zs2a	mouth	11/17/41	sII	−	+	+	+	26A & 96A
Zi2a	intestine	11/19/41	sII	−	+	+	+	26A & 96A
Zs5k	mouth	12/29/41	sI	−	+	+	−	26A & 45A (9A)
Zi5a	intestine	11/18/41	sI	−	+	+	−	26A & 45A (9A)
Zs5f	mouth	12/15/41	sII–sI	+	+	+	−	26A & 96A (19A & 9A)
Zi5f	intestine	12/17/41	sII	+	+	+	−	26A & 96A (19A & 9A)
Zs6e	mouth	12/ 1/41	sI	+	+	+	−, +	26A & 45A
Zi6e	intestine	1/ 2/42	sI	+	+	+	−, +	26A & 45A
Zs6a	mouth	11/17/41	sI	+, −	+	+	+	26A & 96A
Zi6a	intestine	11/18/41	sI	−, +	+	+	+	26A & 96A
Zs13d	mouth	12/15/41	sI	+, −	−	−	−	45A
Zi13c	intestine	12/10/41	sI	+, −	−	−	−	45A
Zs15b	mouth	12/ 8/41	sII	−	+	+	−	26A & 96A
Zi15c	intestine	12/10/41	sII	−	+	+	−	26A & 96A
Zs16g	mouth	12/15/41	iIII	+	−, +	−, +	+	45A
Zi16b	intestine	11/25/41	iIII	+, −	+	−, +	+	45A
Zs16b	mouth	11/24/41	sI	+, −	+	+	−	26A & 96A
Zi16k	intestine	12/23/41	sII–sI	+, −	+	+	−	26A & 96A
Zs16d	mouth	12/ 8/41	iIII–sII	+, −	+	+	−	96A
Zi16e	intestine	12/ 8/41	sII	+, −	+	+	−	96A
Zs18b	mouth	12/ 8/41	sII	+, −	+	+	−	26A & 96A (16A)
Zi18b	intestine	12/10/41	sII	+, −	+	+	−, +	26A & 96A (16A & 45A)
Zs20b	mouth	12/ 8/41	sII	+, −	+	+	+	26A & 96A
Zi20b	intestine	12/10/41	sII	−	+	+	+	26A & 96A

[a] Smooth (s) and intermediate (i) colony form; groups I, II, and III in cell morphology. In three instances the morphology was different before and after the date of carbohydrate extraction. These changes are indicated in the table.

[b] + = acid produced, − = no acid. Tests were usually made within two weeks of time extracts were prepared for immunological tests; if longer time elapsed between fermentation tests and carbohydrate extraction tests were repeated later. When results differed in successive tests both results have been recorded. All reactions in dextrin were negative. All strains produced acid in milk and the 7 other sugars tested.

[c] Secondary, partial cross reactions with antisera shown in parentheses.

relationships could have been missed easily in each cultured sample since the original colonies from which the cultures were started were selected on the basis of morphological differences alone and since only about two-thirds of the cultures that were isolated were studied immunologically.

Actually, these factors were probably of less importance than they appear to be since in several instances cultures were isolated on nearer dates which were probably the same. For example, Zs5a, isolated from saliva collected on November 17, 1941, the day before the stool specimen containing Zi5a was obtained and 6 weeks before the paired Zs5f was isolated from saliva, differed only in that it fermented raffinose and inulin when they did not. This culture was lost, unfortunately, and it was not possible to repeat fermentation tests to determine whether it changed in action on these carbohydrates. Bacterial carbohydrate extracted from it reacted in the same antisera. Also, Zs13c which was isolated from a saliva specimen collected 2 days before the stool specimen from which Zi13c was isolated differed from Zi13c and Zs13d only in the fermentation of arabinose. If these 2 cultures are accepted as being the same as those strains with which they are obviously closely related, there are left only 2 pairs that are widely spaced in time of isolation. Both of these pairs were from patient No. 16. There were 3 intestinal strains and 4 oral strains from this patient that were not studied completely, all of them isolated near the dates of isolation of Zi16e and Zi16k, which may possibly have been as closely related as those discussed above. Oral and intestinal cultures which appeared to be closely related were isolated from each of the other 5 patients but during the period of study these did not become identical in all the characteristics investigated. They were, therefore, not included in Table VII.

Finally, it may be stated that the data shown in Table VII indicate, as did the results of the other comparisons, that the cultures isolated from the mouth and intestine are members of the same species.

DISCUSSION

Many investigators have observed that it is usually difficult to isolate lactobacilli from the intestine of adults unless the subjects are placed on a high carbohydrate diet or are given special milk diets. Lactobacilli can be increased to enormous numbers in the bowel by feeding a diet rich in carbohydrates (8, 40–45), but not if the sugar is introduced directly by injection into the ileum through a fistula (46). These organisms are, however, common constituents of the intestinal flora of children. It is interesting that lactobacilli should be more frequently a part of the intestinal flora at an age when dental caries is most prevalent. This is perhaps a reflection of a higher average lactobacillus content of the saliva. The following facts tend to bear out this relationship. Among the 19 children on whom stool examinations were made the 5 that had lactobacilli in only one-third of the saliva samples provided no stool specimens from which lactobacilli could be isolated. All attempts to isolate lactobacilli from 15 stool specimens, obtained 24 to 48 hours after negative saliva samples had been collected from the same individuals, were unsuccessful. Among the stool specimens

following positive saliva samples, three-fourths yielded lactobacilli when the saliva counts were above 1000 per cc.. and only one-fifth yielded these organisms when the saliva counts were lower. Finally, lactobacilli were not found in any of 14 stool specimens from 5 children whose mouths were free of dental caries. It must be remembered, however, that this relationship between the appearance of lactobacilli in the intestine and the presence of caries-associated lactobacilli in the saliva is not an invariable one. It was reported as long ago as 1900 by Moro (47), Tissier (48) and Finkelstein (49) that microorganisms of this genus are numerous in the intestines of infants.

The question may be raised whether the media employed were so selective for organisms of oral origin that the strains which were isolated from the intestine included only oral organisms that passed through the alimentary tract and not other organisms which might be more properly called intestinal lactobacilli. It is not unlikely that the feces contained strains of lactobacilli that were not recovered in these cultures and, although special effort was made to isolate all the lactobacilli that could be distinguished by colony form, it is not claimed that all the strains that may have been present were isolated in pure culture. There is the possibility that whatever strains may have been missed were all members of a species or group restricted more or less to intestinal habitat, a group not represented in the oral flora. This possibility seems remote, however, and we believe it can be dismissed for the following reasons: (1) Stock cultures of *Lactobacillus acidophilus* originally isolated from the intestine have grown well when tested in the media used in this study. (2) These media have been used successfully in this laboratory for the isolation of lactobacilli from the feces of monkeys when repeated efforts to find them in the mouths of the same animals were made without success, these results having been interpreted by us as evidence that the organisms isolated from the monkey stools could not be merely oral lactobacilli that had been swallowed but probably should be considered as intestinal lactobacilli in the literal sense. (3) The solid medium used was a modification of a tomato agar medium (35) devised by Kulp and used successfully by him and his associates for the isolation of *L. acidophilus* from the intestine. It was made more acid, however, than is usually done for isolation of intestinal lactobacilli. (4) The range of characteristics exhibited by the cultures include the characteristics listed by other investigators in describing intestinal lactobacilli

At any rate there should be little if any doubt that the organisms isolated from the intestine belong to the same group or groups as those obtained from the saliva. In at least 12 instances strains isolated from feces were apparently identical with strains cultured from saliva samples of the same individuals and none of the strains of fecal origin were different in any observed respect from those in the saliva collection. All but 3 of the intestinal strains with which immunological studies have been made reacted type-specifically in antisera prepared from oral lactobacilli. Two of the oral strains also failed to react in these same antisera.

Although no evidence was found that more than 1 species of lactobacilli was represented in the cultures studied, no attempt was made to establish species limits or to make species comparisons with other cultures. For these reasons

we are not able to state definitely whether any or all of our cultures are properly classifiable as *L. acidophilus*, whether they belong to a different species or whether more than 1 species is represented. On the basis of data published by other investigators who have classified the common oral lactobacilli as *L. acidophilus* and because of the findings from several observations made in this laboratory, part of them recorded in this paper, we believe that the first of these 3 possibilities is probably the correct classification.

SUMMARY

Lactobacilli were isolated from saliva and stool specimens from a series of patients hospitalized in a children's hospital for various illnesses unrelated to dental conditions. Cultures were made at intervals over a period of about 6 weeks. At each examination period representatives of each recognizably different type of colony were chosen for subculture and further study. Independent clinical examination of the teeth was made before, during or after the period during which specimens were collected for culturing.

No lactobacilli were found in the stool recimens from 5 children whose teeth were caries free. Lactobacilli were recovered only occasionally and then in small numbers from saliva samples obtained during the same study periods from these 5 children.

Lactobacilli were found in 66% of the fecal specimens and 93% of the saliva samples from 11 individuals who had definite dental caries activity. There was a rough correlation between the number of lactobacilli in the saliva and the frequency of successful isolation of these organisms from the stools.

Approximately 100 cultures obtained from saliva and stool samples provided by 11 of the patients were maintained in laboratory culture media over a period of 16 to 22 months during which time repeated observations were made on gross and microscopic morphology, fermentation capacity and immunological reaction. None of the strains in either the oral or intestinal group exhibited characteristics not found among strains in the other group. Although there were some differences in the proportions of various colony types and fermentation groups at the time of isolation these differences disappeared after a period of laboratory cultivation. More than half of the cultures were tested immunologically in type specific antisera and the same types were found in about the same proportions in the two groups.

Paired, oral and intestinal cultures from 8 individuals, studied comparatively, were found to be identical in morphology, fermentative action and immunological reaction.

It is concluded that the cultures isolated from the intestine had their origin in the mouth and that the supply of these organisms is continually replenished by the swallowing of lactobacillus-contaminated saliva. The relationship of the oral lactobacillus with the intestinal *Lactobacillus acidophilus* is discussed.

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THE EFFECT OF VITAMINS ON ACID FORMATION IN SALIVA[1]

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In 1936 (1) it was suggested that the mechanism by which acids are formed in the mouth may be analogous to the series of chemical reactions that take place during the metabolism of carbohydrates in muscle tissue and in fermentation by various microorganisms (2, 3, 4, 5, 6, 7, 8). Since that time it has been shown that the above suggestion is true (9). Furthermore, it has been shown that certain chemical compounds that are known to inhibit these reactions also inhibit dental caries. More recently it has been demonstrated that the vitamins, particularly those of the B complex, play a very important role in the metabolism of carbohydrates (10). If this is true, it would furnish a theoretical basis for the various clinical reports concerning the effects of vitamins in the development of dental caries.

It has been reported by Kniesner (11) that people suffering from a vitamin B deficiency have very little caries. Other clinical reports indicate that vitamins A, D, and C may influence caries activity (12, 13, 14, 15, 16). On the basis of these reports it was thought that a study of the effect of these vitamins on the rate of acid formation in saliva would be of value.

EXPERIMENTAL

In view of the difficulties involved in producing and estimating acid formation in saliva, the acid formed was determined by its solution effect on human enamel. This eliminates the difficulties encountered by both the titration method and the measurement of pH, and produces conditions more like those which occur in the mouth. As the acids are neutralized by the human enamel as fast as they are formed, there is no drastic increase in the acidity, which would materially inhibit the acid formation by the normal oral bacteria. Furthermore, it furnishes an indication of the acids formed in excess of the normal buffering capacity of the saliva.

Saliva was gathered by paraffin stimulation, well mixed, and divided into several different portions of 10 cc. each. One sample of the fresh saliva was analyzed for calcium. To each of the other samples was added 1 gm. of glucose and 0.1 gm. of 300 mesh powdered human tooth enamel. In addition to this, appropriate amounts of the test material were added to the samples, with the exception of 2 that were used as controls. Two samples of each concentration were made up in order to secure checks. The samples were then sealed and

[1] This work was done under a grant from the Good Teeth Council for Children. Received for publication December 8, 1943.

placed in a water bath and incubated at 37° C. with constant agitation. This constant shaking is essential in order to facilitate the solution of the enamel as fast as the acids are formed. After 4 hours of incubation the samples were removed and analyzed for calcium. This procedure is essentially the same as the chemical test for caries susceptibility (17). The acids formed were expressed as milligrams of calcium dissolved per 100 cc. of saliva.

The effects of beta carotene (vitamin A); thiamin chloride hydrochloride (vitamin B_1); riboflavin (vitamin B_2); pyridoxine (vitamin B_6); pantothenic acid; nicotinic acid; nicotinic amide; para aminobenzoic acid; ascorbic acid (vitamin C); calciferol (vitamin D); alpha tocopherol (vitamin E); 2-methyl-1,4-naphthoquinone; vitamin K_1; and cholesterol were studied. In addition to the above, "Cerophyl," a commercial product, prepared from grass and containing the grass juice factor, was studied.

RESULTS

The results are shown in Table I. It should be noted that the calcium content of the fresh saliva indicates the condition with no acid formed. The results of the control sample indicates the acid formed during the incubation period when no test material is present. Any material deviation from this figure with the use of the test material indicates either a stimulation or inhibition of acid formation. As can be seen from the table, it is evident that under the conditions of the experiment only one of the vitamins, vitamin K, produced a drastic decrease in the rate of acid formation. This compound in a concentration of 1.3 mg./100 cc. caused a complete inhibition of acid formation. There was some evidence that thiamin, nicotinic acid, "Cerophyl" and cholesterol may cause a slight stimulation of acid production.

DISCUSSION

In considering the above results, one should bear in mind the probable mechanisms involved in the production of acids. For this reason a definite stimulation or a definite inhibition indicates a specific effect and should be a criterion of its effect on dental caries. On the other hand, if no effect was observed, it does not mean that the vitamin would have no effect on caries. It is well known that the role of vitamins in carbohydrate metabolism is exceedingly complicated, and that combinations of vitamins with other vitamins or with other compounds or ions may be necessary to cause a stimulation.

It is interesting to note that although checks were made using the saliva from at least 2 individuals with each vitamin, the results of certain of the vitamins varied somewhat from individual to individual. This was particularly true with the members of the B complex and with cholesterol. Thus, it may well be that in those cases in which no effect was observed, or in which variable results were obtained, a stimulation could be produced, provided the proper combinations could be found, or providing a deficiency of any of the vitamins existed. This is demonstrated by the fact that although none of the individual members of the "B complex" caused a marked stimulation at all concentrations, "Cero-

TABLE I

Effect of various vitamins on acid production

TUBE	AMOUNT ADDED		CALCIUM
	mg./100 cc.	*mM./L.*	*mg./100 cc.*
β-Carotene			
Fresh saliva			5.2
1. Control	0	0	24.8
2.	12.5	0.23	23.2
3.	24.0	0.46	21.5
4.	50.0	0.92	20.0
5.	100.0	1.84	22.0
6.	200.0	3.68	20.3
Thiamin			
Fresh saliva			4.5
1. Control	0	0	13.9
2.	14	0.58	19.5
3.	28	1.16	15.6
4.	56	2.32	12.5
5.	112	4.64	13.7
6.	224	9.28	14.7
7.	448	18.56	16.6
Riboflavin			
Fresh saliva			5.2
1. Control	0	0	15.5
2.	14	0.37	15.7
3.	28	0.74	16.5
4.	56	1.48	16.7
5.	112	2.96	16.8
6.	224	5.92	15.9
7.	448	11.84	16.0
Pyridoxine			
Fresh saliva			5.2
1. Control	0	0	24.8
2.	12.5	0.74	21.0
3.	25.0	1.48	18.5
4.	50.0	2.96	20.0
5.	100.0	5.92	20.0
6.	200.0	11.84	18.5
Nicotinic acid			
Fresh saliva			4.8
1. Control	0	0	18.7
2.	14	1.13	24.3
3.	28	2.26	19.4
4.	56	4.52	17.8
5.	112	9.04	18.0
6.	224	18.08	13.2
7.	448	36.16	22.4

TABLE I—*Continued*

TUBE	AMOUNT ADDED		CALCIUM
	mg./100 cc.	*mM./L.*	*mg./100 cc.*
Nicotinic amide			
Fresh saliva			4.3
1. Control	0	0	17.2
2.	14	1.14	17.1
3.	28	2.28	16.5
4.	56	4.56	16.0
5.	112	9.12	22.6
6.	224	18.24	17.3
7.	448	36.48	15.2
p-Aminobenzoic acid			
Fresh saliva			7.5
1. Control	0	0	23.5
2.	10	0.73	21.5
3.	20	1.46	21.0
4.	40	2.92	18.5
5.	80	5.84	18.5
6.	120	8.76	17.5
7.	160	11.68	17.8
8.	200	14.60	16.5
Calcium pantothenate			
Fresh saliva			7.5
1. Control	0	0	23.5
2.	10	0.19	23.5
3.	20	0.38	22.0
4.	40	0.77	21.5
5.	80	1.55	19.0
6.	120	2.33	19.0
7.	160	3.10	19.0
8.	200	3.88	18.0
Ascorbic acid			
Fresh saliva			5.3
1. Control	0	0	24.8
2.	12.5	0.71	21.5
3.	25.0	1.42	21.0
4.	50.0	2.84	21.5
5.	100	5.68	21.5
6.	200.0	11.36	21.5
Viosterol			
Fresh saliva			5.3
1. Control	0	0	24.8
2.	12.5	0.32	21.8
3.	25.0	0.64	20.7
4.	50.0	1.28	17.5
5.	100.0	2.56	18.5
6.	200	5.12	24.0

TABLE I—*Continued*

TUBE	AMOUNT ADDED		CALCIUM
	mg./100 cc.	*mM./L.*	*mg./100 cc.*
α-Tocopherol			
Fresh saliva			5.3
1. Control	0	0	24.8
2.	12.5	0.29	22.5
3.	25.0	0.58	22.0
4.	50.0	1.16	21.2
5.	100.0	2.32	16.0
6.	200.0	4.64	22.5
2-Methyl-1,4-naphthoquinone			
Fresh saliva			5.6
1. Control	0	0	26.6
2.	1.35	0.078	4.8
3.	2.30	0.133	4.7
4.	3.50	0.203	2.6
5.	8.00	0.465	2.5
6.	11.30	0.658	2.6
7.	18.50	1.075	2.4
Vitamin K_1			
Fresh saliva			5.5
1. Control	0	0	25.0
2.	500	11.12	11.0
Cerophyl			
Fresh saliva			
1. Control	0		23.2
2.	10		27.5
3.	50		29.0
4.	100		37.5
Cholesterol			
Fresh saliva			5.8
1. Control	0	0	10.0
2.	25	0.64	14.0
3.	50	1.29	17.5
4.	125	3.24	18.0
5.	250	6.48	18.0
6.	500	12.96	18.0

phyl," which is composed of all known members of the "B complex" group (including the grass juice factor) and vitamins A and C, caused a significant stimulation of acid formation. This may explain the results of Kniesner, Mann and Spies, who found a decreased caries activity in patients suffering from a vitamin B deficiency, but could not find a significant difference in the salivary concentration of certain members of this group of vitamins. Although it is probable that these results on individual vitamins may not indicate stimulating effects of certain combinations, it is also possible that combinations may produce inhibiting effects. This is extremely unlikely, however, as all of the known inhibitors at present are single compounds.

One should also keep in mind that the saliva used in these experiments was taken from dental students who were in good health but who were caries active individuals. The diet of these individuals was adequate, and hence it would be expected that normal amounts of all of the vitamins would be present in the saliva. Insofar as the vitamins are usually active in very small quantities, it is quite possible that the optimum amount of each was present in the original samples. However, as it would be impossible to remove these from saliva, no attempt was made to do so.

In view of the fact that the synthetic vitamin K completely inhibited the reaction, it would be interesting to know if this action has any relation to its vitamin K activity. For this reason, some of the extremely unstable natural vitamin K_1 was synthesized and tested. Due to the cost and difficulty of synthesis of this compound, the effect was tried in only 1 concentration—500 mg. per 100 cc. A definite but incomplete inhibition was observed. The 500 mg. per 100 cc. caused an inhibition of over 50%. This would indicate that the 2 actions are not closely related. Furthermore, we have since tested many other anti-oxidants, several of which were also tested by Armstrong (18). Many of these compounds act similarly to 2-methyl-1,4-naphthoquinone.

Cholesterol, which is now considered as having vitamin properties, usually showed a stimulation of acid production. It is interesting to note that Krasnow (19) found a definite trend toward high cholesterol concentrations in the saliva of caries active individuals.

SUMMARY

The effect of most of the known vitamins on acid formation from fermentable sugars in saliva has been studied. Only cholesterol, "Cerophyl", a mixture of vitamins, and possibly thiamin stimulated acid formation. Synthetic vitamin K (2-methyl-1,4-naphthoquinone) and natural vitamin K_1 (2-methyl-3-phythl-1,4-naphthoquinone) inhibited acid formation. There was no indication that this action has any relationship to its vitamin K activity.

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THE EFFECT OF SYNTHETIC VITAMIN K AND RELATED COMPOUNDS ON THE RATE OF ACID FORMATION IN SALIVA[1]

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It has been shown that synthetic vitamin K (2-methyl-1,4-naphthoquinone) is among the few vitamins that influence acid formation in fermenting saliva-sugar solutions (1). Insofar as this compound has a marked inhibitory effect, it was suggested that it may have some therapeutic value in the prevention of dental caries (2). As this effect is probably not associated with its vitamin activity (1, 3), it was thought of interest to study compounds of similar molecular configuration and compounds which may act in oxidation-reduction systems.

Armstrong (3) suggested that the effect is due to its bactericidal properties, but it was found in this laboratory that the inhibition of acid formation could be detected in concentrations that had no effect on the bacterial flora of the saliva. It is quite probable that the substance acts as an enzyme inhibitor, and that the inhibition would be effective in concentrations which would not necessarily kill the organisms.

As the synthetic vitamin K is a quinone, it was thought that it might possibly interfere with the normal oxidation-reduction potential of the fermenting mixture and thus either prevent the normal fermentation process or cause the reactions to proceed in other directions.

The compounds tested were 1,4-quinone; hydroquinone; 2-methyl-1,4-naphthoquinone; vitamin K_1; "menadione sodium bisulfite"; "Hykinone" (the sodium bisulfite addition compound with 2-methyl-1,4-naphthoquinone); "Synkayvite" (tetrasodium-2-methyl-1,4-naphthohydroquinone phosphate); 2,3 dichloro-1,4-naphthoquinone; 2-methyl-1,4-naphthohydroquinone; 2-methyl-1,4-naphthohydroquinone diacetate; potassium 2-methyl-1,4-napthohydroquinone-3-sulfonate; "Styptavite" (a 2-methyl-1,4-naphthoquinone salicylic acid mixture or complex); citrinin; l-cystine; alloxan; sodium malonate; ethyl malonate; sodium maleate; morpholine; p-aminophenylglycine; hydrogen peroxide; sodium carbonate peroxide; urea peroxide; magnesium peroxide; and urethane.

EXPERIMENTAL

The experimental procedure was exactly the same as when the vitamins were used as the test materials (1). Saliva was gathered by paraffin stimulation, and

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TABLE I

TUBE	AMOUNT ADDED		CALCIUM
	mg./100 cc.	*mM./L.*	*mg./100 cc.*
1,4-Quinone			
Fresh saliva			3.5
1. Control	0	0	17.7
2.	14	1.30	4.4
3.	28	2.60	4.1
4.	56	5.20	3.6
Hydroquinone			
Fresh saliva			6.0
1. Control	0	0	22.0
2.	10	0.91	12.0
3.	20	1.82	14.0
4.	40	3.64	5.3
2-Methyl-1,4-naphthoquinone			
Fresh saliva			5.6
1. Control	0	0	26.6
2.	1.35	0.078	4.8
3.	2.30	0.133	4.7
4.	3.50	0.203	2.6
5.	8.00	0.465	2.5
6.	11.30	0.658	2.6
7.	18.50	1.075	2.4
Vitanin K_1			
Fresh saliva			5.5
1. Control	0	0	25.5
2.	500	11.12	11.0
Hykinone			
Fresh saliva			7.5
1. Control	0	0	18.5
2.	10	0.36	4.1
3.	20	0.72	4.0
4.	40	1.44	4.0
Synkayvite			
Fresh saliva			7.0
1. Control	0	0	15.0
2.	10	0.19	15.0
3.	30	0.57	15.0
4.	50	0.95	7.0
5.	100	1.90	6.5
6.	200	3.80	6.5
2,3-Dichloro-1,4-Naphthoquinone			
Fresh saliva			8.5
1. Control	0	0	16.0
2.	10	0.44	3.9
3.	20	0.88	2.9
4.	40	1.76	2.7
5.	80	3.52	2.5
6.	160	7.04	1.8
7.	320	14.08	1.6

TABLE I—*Continued*

TUBE	AMOUNT ADDED		CALCIUM
	mg./100 cc.	*mM./L.*	*mg./100 cc.*
2-Methyl-1,4-Naphthohydroquinone			
Fresh saliva			7.5
1. Control	0	0	17.0
2.	10	0.57	4.5
3.	20	1.15	4.0
4.	40	2.30	3.5
5.	80	4.60	3.5
6.	120	6.90	3.3
7.	160	9.20	3.5
2-Methyl-1,4-Naphthohydroquinone Diacetate			
Fresh saliva			4.5
1. Control	0	0	13.0
2.	20	0.78	8.0
3.	40	1.55	7.5
4.	60	2.32	7.5
5.	80	3.10	7.2
6.	100	3.88	7.9
7.	120	4.64	7.4
Potassium 2-Methyl-1,4-Naphthohydroquinone-3-Sulfonate			
Fresh saliva			6.4
1. Control	0	0	16.5
2.	10	0.34	15.5
3.	20	0.69	12.0
4.	40	1.39	12.0
5.	60	2.06	9.0
6.	80	2.74	7.0
7.	100	3.43	6.0
Styptavite			
Fresh saliva			4.0
1. Control	0		14.5
2.	10		17.5
3.	20		13.5
4.	40		9.5
5.	80		4.0
6.	160		4.0
Citrinin			
Fresh saliva			6.5
1. Control	0		13.0
2.	10		6.5
3.	20		3.5
4.	40		3.5
5.	80		3.0

TABLE I—*Continued*

TUBE	AMOUNT ADDED		CALCIUM
	mg./100 cc.	*mM./L.*	*mg./100 cc.*
Menadione Sodium Bisulfite			
Fresh saliva			7.1
1. Control	0	0	27.3
2.	10	0.36	4.0
3.	20	0.72	3.8
4.	40	1.44	3.9
5.	60	2.16	3.5
6.	80	2.88	3.3
7.	120	4.32	4.2
l-Cystine			
Fresh saliva			2.1
1. Control	0	0	25.0
2.	10	0.42	26.0
3.	20	0.84	36.0
4.	40	1.68	27.6
5.	80	3.36	23.4
6.	160	6.72	21.0
7.	320	13.44	25.0
Alloxan			
Fresh saliva			5.0
1. Control	0	0	23.0
2.	10	0.70	12.1
3.	20	1.40	11.2
4.	40	2.80	11.5
5.	60	4.20	11.5
6.	80	5.60	12.0
7.	100	7.00	11.0
8.	120	8.40	10.5
Sodium Malonate			
Fresh saliva			4.5
1. Control	0	0	14.2
2.	10	0.67	14.0
3.	20	1.35	14.0
4.	40	2.70	8.2
5.	80	5.40	8.0
6.	160	10.80	8.0
7.	320	21.80	8.0
Ethyl Malonate			
Fresh saliva			7.0
1. Control	0	0	21.5
2.	10	0.62	18.5
3.	20	1.25	18.0
4.	60	3.75	7.0
5.	100	6.25	6.5
6.	120	7.50	6.5

TABLE I—*Continued*

TUBE	AMOUNT ADDED		CALCIUM
	mg./100 cc.	*mM./L.*	*mg./100 cc.*
Sodium Maleate			
Fresh saliva			2.1
1. Control	0	0	25.0
2.	10	0.63	19.0
3.	20	1.25	24.4
4.	40	2.50	17.7
5.	80	5.00	25.2
6.	160	10.00	17.9
7.	320	20.00	14.6
Morpholine			
Fresh saliva			5.5
1. Control	0	0	25.5
2.	1	0.11	25.5
3.	10	1.14	25.5
4.	100	11.49	24.0
p-Aminophenylglycine			
Fresh saliva			6.0
1. Control	0	0	23.0
2.	10	0.60	23.0
3.	50	3.01	21.0
4.	100	6.02	18.5
5.	150	9.03	17.0
6.	200	12.04	15.0
Hydrogen Peroxide			
Fresh saliva			6.5
1. Control	0	0	29.5
2.	10	2.94	24.0
3.	20	5.88	22.5
4.	40	11.76	18.0
5.	60	17.64	14.5
6.	80	23.52	13.0
7.	100	29.40	13.5
Sodium Carbonate Peroxide			
Fresh saliva		*	4.5
1. Control	0	0*	13.0
2.	10	0.44*	7.1
3.	20	0.88*	6.5
4.	30	1.32*	5.8
5.	40	1.76*	7.5
6.	50	2.20*	6.5
7.	60	2.64*	6.3
8.	80	3.52*	6.0
9.	120	5.28*	5.3

* Millimols of active oxygen.

TABLE I—*Continued*

TUBE	AMOUNT ADDED		CALCIUM
	mg./100 cc.	*ml./L.*	*mg./100 cc.*
Urea Peroxide			
Fresh saliva		*	8.5
1. Control	0	0*	21.5
2.	20	1.0*	9.5
3.	40	2.0*	6.5
4.	60	3.0*	5.5
5.	80	4.0*	5.5
6.	100	5.0*	4.8
7.	120	6.0*	4.0
8.	140	7.0*	3.2
Magnesium Peroxide			
Fresh saliva		*	6.5
1. Control	0	0*	22.0
2.	10	0.44*	20.0
3.	20	0.88*	20.0
4.	40	1.76*	20.0
5.	60	2.64*	6.5
6.	80	3.52*	6.5
Urethane			
Fresh saliva			5.1
1. Control	0	0	23.0
2.	10	1.12	20.5
3.	20	2.24	20.5
4.	40	4.48	21.0
5.	60	6.72	20.0
6.	80	8.96	19.5
7.	100	11.20	17.5
8.	120	13.44	17.5

after the addition of the test materials, sugar and enamel, was incubated for 4 hours in a thermoregulated water bath at 37°C. The usual controls were run simultaneously. The difference in the calcium content of the test samples and the controls is a function of the acids formed. The results are in Table I.

RESULTS

It should be noted that the calcium content of the fresh saliva represents the control level. The results of the control sample indicate the acid formed during the incubation period when no test material is present. Any marked variation from this figure with the use of the test material indicates either an inhibition or a stimulation of acid formation.

As can be seen from the above table, all of the derivatives of the quinones are effective in inhibiting acid formation. This applies not only to the quinones but also to the reduced compounds such as hydroquinone and potassium 2-methyl-1,4-napthohydroquinone-3-sulfonate, and the phosphoric ester of the reduced quinone—"Synkayvite". It is possible that an equilibrium was reached between the oxidized and reduced forms while in the saliva, as this is assumed to take place when these compounds are used as the vitamin, and it is supposed

that the vitamin K activity is related in some way to the redox system. Citrinin, a compound produced by Penicillium citrinum, may also be placed in this class, as it has been established that it is structurally similar to the quinones (4). This compound also inhibits acid formation.

In addition to the derivatives of the quinones, several compounds that are known to inhibit the activity of dehydrogenases (5, 6) and esterases (7) were tried. In general these compounds did not appreciably inhibit acid formation until very high concentrations were reached. With ethyl and sodium malonate and urethane, some inhibition was observed in the higher concentrations. l-Cystine, which is supposed to take part in certain oxidation reduction reactions, showed no appreciable effect on these reactions.

Certain compounds, such as morpholine, p-amino phenylglycine, and sodium maleate, have been used for some time as anti-oxidants in the rubber industries. For this reason these compounds were tested. None of these compounds showed any appreciable effect on acid formation, with the exception that in the higher concentrations a slight inhibition was observed.

In the past few years, several new peroxides have been placed on the market, so it was thought interesting to try these compounds in conjunction with hydrogen peroxide. It should be noted that hydrogen peroxide had very little effect on the rate of acid formation. Magnesium peroxide had little effect in the lower concentrations, but sodium carbonate peroxide and urea peroxide were quite effective. Insofar as Stephan (8) has suggested that urea may be used as an inhibiting agent for dental caries, the action of urea peroxide may be twofold.

On the basis of the above experiments, the only compounds found which would materially inhibit acid formation were the quinones and derivatives and the peroxides. In general, the other compounds had little effect or were effective only in high concentrations.

SUMMARY

The effect of certain quinones and derivatives, and certain other compounds which may affect the oxidation reduction systems involved in the formation of acids by fermentation, have been tested for their effect on acid production in saliva. Of those tested, 2-methyl-1,4-naphthoquinone was found equal to any others tested, and superior to most of them. Aside from the quinones and derivatives, the only other compounds tested which were particularly effective were the various peroxides.

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THE COURSE OF THE ENAMEL PRISMS NEAR THE CEMENTO-ENAMEL JUNCTION[1]

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The histologic study of enamel is considered difficult because observations have been made chiefly on ground sections. According to Lehner and Plenk (1), Sudduth was first to demonstrate the feasibility of decalcifying enamel embedded in celloidin. Bodecker (2) introduced the simultaneous decalcification and embedding technic, but the method remained limited, permitting study of pieces of enamel but not of the whole tissue or the course of the prisms. Nevertheless, Diamond and Weinmann (3) were able to find that where maturation is about to take place, the enamel matrix is completed.

Gottlieb (4) described prism differentiation as the second stage of matrix formation typified in hematoxylin-eosin stained preparations. In its early stage of formation, the matrix stains dark, not permitting discernment of different structures in the tissue. In later stages, interprismatic threads retain their dark tincture, while strips between them gradually assume a lighter stain apparently because of additional calcification. Thus the prisms, separated by darker staining interprismatic threads, become visible. In decalcification the acid-resistant matrix remains intact, permitting its study.

Black (5) studied the course of the enamel prisms in ground sections after etching the enamel with acid. He reported that the enamel prisms near the cemento-enamel junction in deciduous teeth form an angle with the dentin opening occlusalward, while in permanent teeth, the angle opens apically. Lehner and Plenk (1) observed in ground sections that the prisms at the cemento-enamel junction course perpendicularly to the tooth axis.

MATERIAL

Examinations were made of the enamel in 110 decalcified longitudinal sections of tooth germs in the matrix stage of enamel formation. No specimen was accepted for study which did not allow discernment of individual prisms. In addition, 124 ground sections of fully calcified enamel were studied to check the observations made in the sections of developing enamel. Routine procedures were employed in the preparation of this material.

FINDINGS

Of the 110 sections of enamel studied, in the matrix stage the course of the prisms at the cemento-enamel junction, visualized to their most peripheral end, formed an acute angle with the dentin opening occlusally (prisms in occluso-

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Fig. 1. Decalcified section of maxillary first incisor tooth germ. (D) dentin. (E) enamel matrix in stage of prism differentiation. (M) enamel in stage of maturation.

Fig. 2. Higher magnification of tooth germ in *fig. 1* showing enamel (E) at cemento-enamel junction. Newly differentiated prisms lie in occluso-angular position. (D) Dentin.

Fig. 3. Decalcified section of canine tooth germ. (D) Dentin; (E) enamel matrix in stage of prism differentiation; (M) mature enamel.

angular position, *figs. 1, 2, 3, and 4*) in 95 and coursed at right angles to the long axis of the tooth (*fig. 5*) in 7. In the remaining 8, the rod segments adjacent to

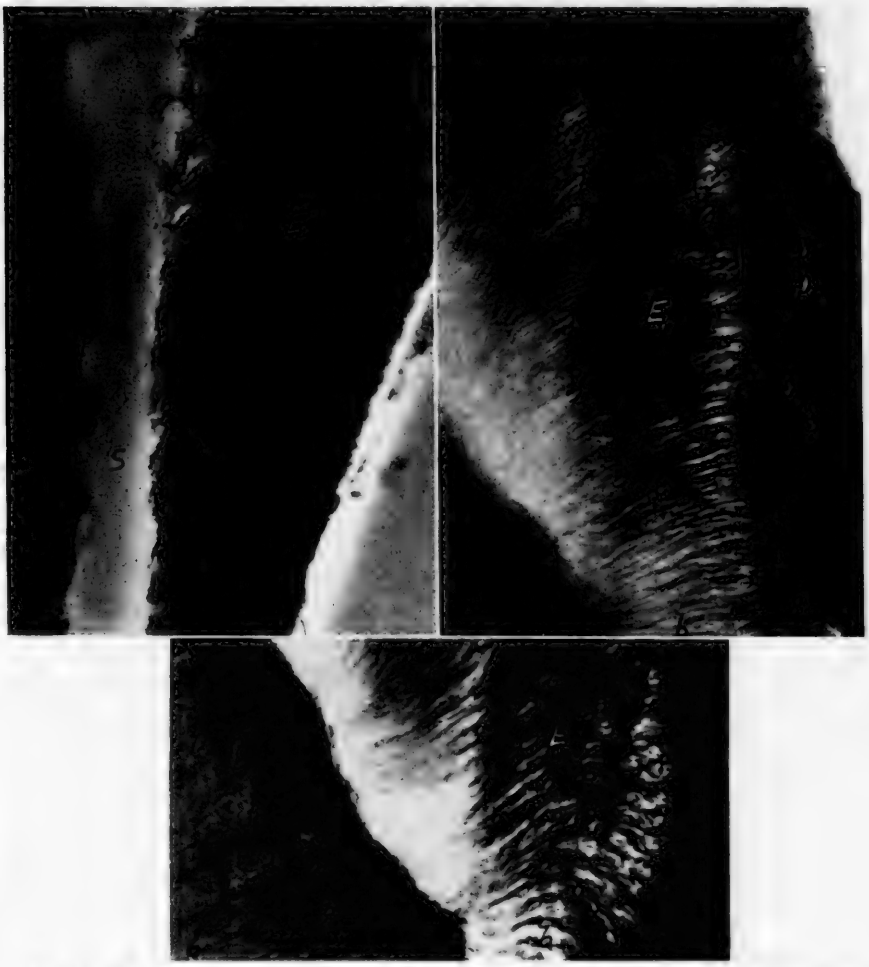

Fig. 4. Higher magnification of tooth in *fig. 3* showing area near cemento-enamel junction. Entire enamel (E) shown is probably not in stage of prism differentiation but rods may be seen forming acute angle with dentin opening occlusalward. (D) dentin; (S) space due to shrinkage in histological preparation.

Fig. 5. Decalcified section of tooth germ showing area near cemento-enamel junction. (E) Enamel matrix, (a) prisms in occluso-angular position; (b) prisms lying at right angles to long axis of tooth. The matrix is mature and calcification has begun.

Fig. 6. Decalcified section (orig. mag. × 800) of tooth germ showing area near cemento-enamel junction. (E) Enamel matrix; (a) prisms in occluso-angular position, (b) prisms adjacent to cemento-enamel junction arched toward root apex; (D) dentin.

the dentin coursed angularly in an occlusal direction, but peripherally, the prisms changed their course to one directed apically (*fig. 6*).

In 79 of the 124 ground sections, the prisms near the cemento-enamel junction occupied a position approximately 90° to the long axis of the tooth. Thirty-seven revealed an occluso-angularly directed course of the prisms. In 8, the rods assumed an apically directed course from the dento-enamel junction. A synopsis of the findings is shown in Table I.

Enamel immediately occlusal to the cemento-enamel junction in decalcified sections often revealed prisms directed occlusally or bent apically in ground sections of the same tooth. Occasionally, the prisms were inclined in an occlusal direction on one side, while on the opposite side of the same ground section, they would be directed apically. Groups of cross sectioned prisms were rarely found near the cemento-enamel junction. As a rule, in longitudinal tooth sections the prisms assumed parallel wave-like courses in a plane parallel to the cut surface.

TABLE I

The position of enamel prisms adjacent to the cemento-enamel junction

	110 DECALCIFIED SECTIONS OF ENAMEL IN MATRIX STAGE	124 GROUND SECTIONS OF ENAMEL FULLY CALCIFIED
90° to long axis of tooth	7 (6%)	79 (64%)
Occluso-angular	95 (87%)	37 (30%)
Arched toward root apex	8 (7%)	8 (6%)

DISCUSSION

Indication of an occluso-angular position of the prisms in a large number of sections of enamel in the stage of prism differentiation seemed unreliable and was the reason for checking the findings in ground sections of mature teeth. In them was found a considerably greater proportion of prisms coursing perpendicularly to the long axis of the tooth, confirming previous observation (1).

The statement of Diamond and Weinmann (3) that "there is complete matrix formation at the beginning of maturation of the enamel at the occlusal surface" is not to be understood as meaning there is completion of the formation of the matrix at the cemento-enamel junction in this phase of tooth development. On the contrary, there is completion only of that part of the matrix adjacent to the point of beginning maturation. At this time the surface of the primary dentin at the cemento-enamel junction is usually scalloped, indicating enamel matrix formation. It is likely, then, that even where a considerable part of the occlusal enamel is already mature, the entire matrix may not be complete.

Such a finding would account for the difference in the courses of the prisms near the cemento-enamel junction in the matrix stage and in ground sections. The change in the course of the prisms from occluso-angular to one perpendicular to the long axis of the tooth, when observed, was probably accomplished during the final stage of formation of the peripheral end of the enamel rods. It may be reasonable to postulate that this alteration could be the result of forces which ultimately cause eruption of the tooth, though no evidence to this effect is available.

The period of time elapsing between the developmental stages of homogeneity, prism differentiation, and maturation are not known, but it would appear that maturation begins very soon after local completion of the matrix. Hence, only a short time is available for accurate study of that tissue in the stage of prism differentiation or prior to possible dislocation of the rods during maturation. A stage where the entire enamel matrix reveals differentiated prisms without evidence of maturation does not seem to exist. At the same level of a given section there may be parts of the matrix in a stage of clearly differentiated prisms adjacent to the dentin while the peripheral enamel appears homogeneous and without evidence of differentiation.

Study of the course of the prisms in the matrix stage may also be modified by variation in the amount of organic material in different enamel tissues. Where a comparatively large residue of organic material is present in the enamel, as in that adjacent to the cemento-enamel junction, the prisms are better studied in specimens decalcified and stained routinely than in those prepared by special technics.

SUMMARY

The enamel prisms in the region of the cemento-enamel junction, as seen in sections of enamel in the stage of prism differentiation, usually occupy an occluso-angular relationship with the dentin. This finding was not confirmed in an investigation of ground sections of fully calcified enamel wherein the number of specimens revealing an occluso-angular relationship between rods and dentin did not exceed 30% of the total. The discrepancy appeared to be the result of a change in the course of the prisms from an occluso-angular to one at right angles to the long axis of the tooth, evidently occurring during the stage of enamel maturation. Occasionally, the enamel rods near the cemento-enamel junction may lie in an arc, the peripheral end of which is directed toward the root apex. Prisms directed apically and occluso-angularly may occur at various places along the cemento-enamel junction of the same tooth.

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THE EPITHELIAL SHEATH: ITS FUNCTIONAL CYCLE, PART II[1]

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As has been shown in a previous study (1), the histogenesis of the epithelial sheath of the human tooth can be observed only at the stage in development at which the cervical enamel is still in the process of formation and the enamel organ is still intact in the cervical region. At a later stage, when the enamel is completely formed and the coronal two-thirds of the enamel is fully calcified, the root dentin and cementum are in the process of formation; and the histogenesis of the epithelial sheath can no longer be observed. Instead, at this early functional state of the epithelial sheath one gets a false impression regarding its histogenesis. Mummery's conclusions (2), that the cells of the epithelial sheath "are probably derived from proliferations of the tooth band within the folicle, and possibly also from the buddings or proliferations of the external epithelium of the enamel organ," are based upon observations made upon a human tooth at such a stage of development, when the epithelial sheath was already in a functional state and the organic connection between the ameloblasts and the inner layer of the epithelial sheath was disrupted.

FINDINGS

Fig. 1 is a low power view of a human mandibular deciduous second molar at a stage of development at which the root dentin (RD) is in an early state of formation. The enamel is completely formed. The coronal two-thirds of the enamel (E) is fully calcified and the enamel (M) in the cervical region is still in a matrix state. This specimen is stained with hematoxylin-eosin. The area enclosed within the square is represented in higher magnification in *fig. 2*. *Fig. 2* shows the cervical enamel in a matrix state at (M), terminating at the cervical line (CL) where it joins a thin layer of cementum (C). The epithelial sheath is seen at (EP). The area enclosed within the square is represented in higher magnification in *fig. 3*. *In fig. 3* the enamel is seen to extend as far as the cervical line (CL). The ameloblasts (A) appear as large oval cells in close contact with the periphery of the enamel matrix. These cells are seen to terminate at the cervical line (CL), where the enamel matrix terminates. Adjacent to the ameloblasts at (R) are several closely compact layers of flattened elongated cells. These layers appear widely separated in the region (L) as they extend beyond the cervical line in the direction of the root. Several rounded cells (DE) are seen lying between the layer of cementum (C) and the elongated flattened epithelial cells. (CB) are cementoblasts. *Fig. 4* is a higher magnification of an area

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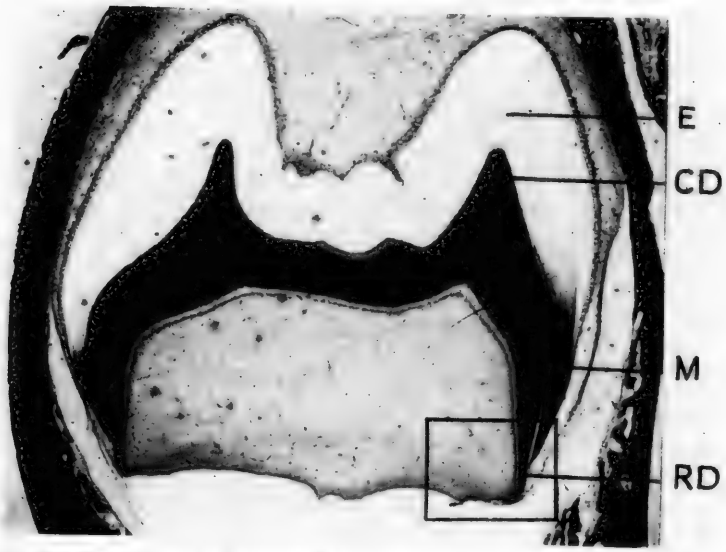

Fig. 1. Low power view of developing mandibular deciduous second molar at stage in which root of tooth is at beginning of formation. E—fully calcified enamel; CD—crown dentin; M—enamel matrix in cervical region; RD—root dentin. Area enclosed within square is presented in higher magnification in *fig. 2*. Specimen from collection of Dr. Isaac Schour.

Fig. 2. D—dentin; PD—predentin; O—odontoblasts; P—pulp; M—enamel matrix; C—cementum; CL—cervical region or cemento-enamel junction; EP—epithelial sheath. Area enclosed within square is presented in higher magnification in *Fig. 3*.

similar to the one in *fig. 3*, taken from another section of the same series as *fig. 1*. (M) is the enamel matrix. (A at CL) the ameloblasts at cervical line. The cells of outer enamel epithelium are indicated as (R) and the cemento-enamel junction as (CL). (DE) is a large rounded or oval cell, one of a layer of cells extending from the ameloblasts at the cemento-enamel junction. (CB) are the cementoblasts, (I) is the inner layer of the epithelial sheath, (O) the outer layer of the epithelial sheath and (C) is the cementum.

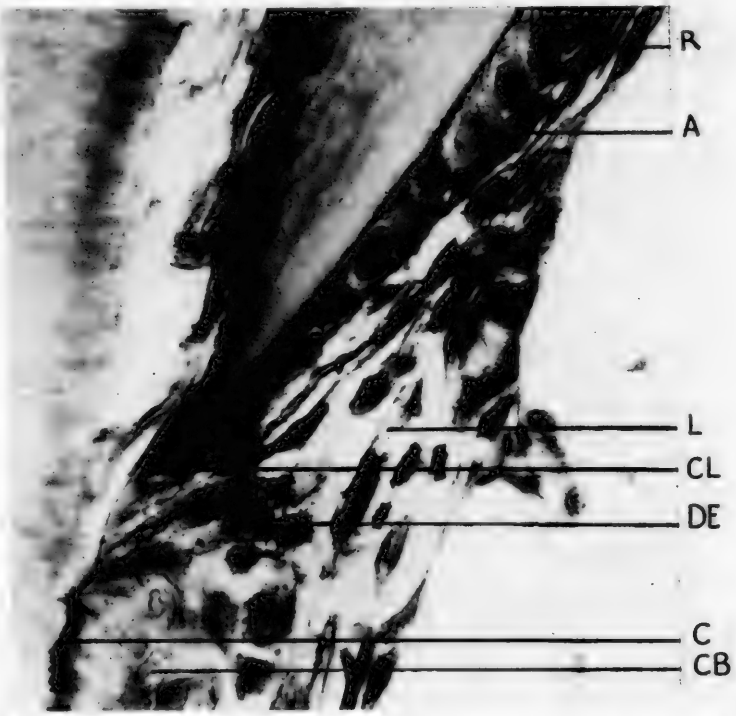

Fig. 3. R—reduced cells of enamel organ; A—ameloblasts; L—loose strands of reduced outer enamel epithelium; CL—cemento-enamel junction where ameloblasts terminate; DE—degenerating cells of inner layer of epithelial sheath; C—cementum; CB—cementoblasts.

DISCUSSION

An examination of the photomicrographs in the stage of the life cycle of the epithelial sheath represented in *fig. 3* gives the illusion that the epithelial sheath is derived solely from the external epithelium of the enamel organ. The ameloblasts (A) in *fig. 3*, still evident adjacent to the enamel matrix in the cervical region, are generally oval in form; this is characteristic of their reduced or degenerating state. They extend as far as the cervical region of the crown where the enamel matrix terminates at (CL) (*fig. 3*), and seem to have no organic connection with the epithelial sheath.

The markedly flattened and reduced stellate cells and the cells of the outer enamel epithelium lie in several layers adjacent to the reduced ameloblasts at (R) in *fig. 3*. From this region these cells may be followed in loose strands in the direction of the epithelial sheath, with which they still seem to retain an organic connection.

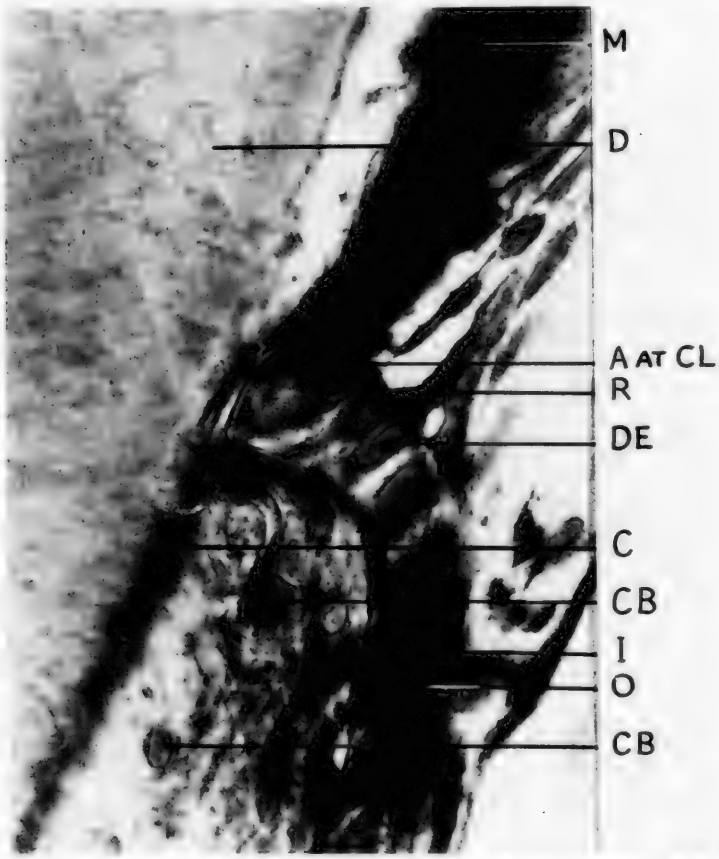

Fig. 4. High power magnification of same general area as that shown in *fig. 3*, taken from another section of same series as *fig. 1*. M—enamel matrix; D—crown dentin; A at CL—ameloblasts at cemento-enamel junction; R—strand of reduced outer enamel epithelium; DE—degenerating cells of inner layer of epithelial sheath; C—cementum; CB—cementoblasts; I—inner layer of epithelial sheath; O—outer layer of epithelial sheath. Epithelial sheath follows curve from ameloblasts at cemento-enamel junction.

The evidence at this stage of development of the tooth tends to support the observation of Mummery that the inner enamel epithelial cells play no part in the histogenesis of the epithelial sheath, for these cells are seen to terminate at the cervical region of the crown. Only the outer enamel epithelial cells are seen to extend in the direction of the epithelial sheath and to maintain organic con-

nection with it. These findings, however, represent only a transitory stage in the life cycle of the epithelial sheath and cannot be accepted out of context with its entire life cycle, for at this stage the organic connection between the ameloblasts and the inner layer of the epithelial sheath is disrupted. A closer examination even at this stage still gives some evidence of organic connection between the ameloblasts and the inner layer of the epithelial sheath. The few isolated cells (DE) in *fig. 3* are remnants of the inner layer of the epithelial sheath. These cells extend to the cervical border of the ameloblasts.

Fig. 4 more strikingly presents the evidence of organic connection between the ameloblasts and the inner layer of the epithelial sheath. *Fig. 4* represents the same region as that shown in *fig. 3*, but is taken from another section of the same developing tooth. The degenerating isolated cells (DE), of the inner layer of the epithelial sheath seen in *fig. 3*, are in this section (*fig. 4*) still within the basal and peripheral limits of their membrane. This layer of cells in the process of degeneration extends from the cervical terminal end of the ameloblasts at almost right angles to the long axis of the tooth, and then curves apically until it is almost parallel to the cementum. The entire extent of the layer is some distance removed from the cementum, having been pushed aside by the invasion of connective tissue which is profusely interspersed with cementoblasts. The loose strands (R) of the outer enamel epithelium are still joined to the peripheral end of the inner layer. At (O) the outer epithelial layer lies adjacent to the inner epithelial layer; together they constitute the epithelial sheath.

The true histogenesis of the epithelial sheath can be observed only in an earlier stage of development, when the enamel matrix is still in the process of formation and the enamel organ is still intact in the cervical region. This was demonstrated in a previous publication (1) in which adjacent layers of "inner and outer enamel" epithelial cells were shown to have originated from the region of confluence of the inner and outer enamel epithelial cells of the enamel organ.

It was maintained that this region of confluence was the future cervical region of the crown. It was maintained, too, that the true inner enamel epithelial cells terminate at this region. The continuing layer of cells forming the inner layer of the epithelial sheath, while they resemble morphologically the undifferentiated state of the ameloblasts, possess, in fact a totally different pattern of morphologic and histochemical characteristics. These cells do not undergo the morphologic changes characteristic of the ameloblasts in their differentiating stages, nor are they secreting cells.

These general conclusions are supported by the evidence in *fig. 3* and more strikingly in *fig. 4*, where the ameloblasts terminate at the cervical region; in *fig. 3*, the organic connection with the inner layer of cells of the epithelial sheath is lost. At this early stage of root formation, with the peripheral layers of dentin formed and covered by the beginning layers of cementum, the inner cells of the epithelial sheath in this region have completed their functional usefulness and are already degenerating.

It may be observed that, just as development occurs in a progressive manner, so the breaking down or degeneration of the scaffolding cells occurs progressively

as their functional cycle is completed. This is first evidenced with the cells of the enamel organ in the outermost incisal or occlusal region of the crown, and continues progressively until the root is completed, when, with the exception of epithelial rests, there is no longer any evidence of an enamel organ or epithelial sheath.

CONCLUSION

The life cycle of the epithelial sheath cannot be observed in any one stage of the development of the tooth. Its true histogenesis may be observed only at a time when the enamel matrix is still in the process of formation in the cervical regions. At a later stage in development, when the root dentin and cementum are in the process of formation, the organic connection between the epithelial sheath and the inner and outer layers of the enamel organ can no longer be seen. A false view regarding the histogenesis of the epithelial sheath may be readily obtained at this stage.

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SOME OBSERVATIONS ON THE ELIMINATION OF YEAST FROM THE MOUTH[1]

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Knighton's findings (1) that certain food stuffs seem to be more effective in cleaning the teeth than others and that the generally practiced method of mouth hygiene is relatively ineffective offers strong support for the ideas of Sim Wallace (2) and Pickerill (3) who have strenuously maintained that one of the best methods of cleaning the teeth was to take advantage of the cleansing action of certain foodstuffs. Knighton's work is important not only because it yields valuable information on the cleansing capacity of various foodstuffs which can be applied directly to caries' prophylaxis, but also because it provides an experimental approach to a neglected field of study. The expansion of this approach and the use of improved experimental procedures could well produce results which would revolutionize conventional ideas on caries control. As the first step in this direction it seemed worthwhile to check Knighton's experiments and whenever posever possible to expand them.

EXPERIMENTAL METHODS

The methods used were essentially those developed and described by Knighton. The effectiveness of various cleansing procedures in the mouth was measured by determining the numbers of yeast cells recovered after eating a yeast cake. Yeast counts were made by having the subject chew a piece of paraffin weighing 2.5 grams for 3 minutes, collecting the stimulated saliva in a sterile test tube and making a yeast count. This was done by plating 0.1 c.c. of suitable dilutions of saliva on Sabouraud's agar and incubating for 48 hours. Such counts were made at the beginning of each experiment (initial yeast count) to determine the number of yeasts present before the experiment. Control yeast elimination figures were then worked out for each subject by making 3 counts 2 hours after the chewing of the yeast cake. To determine the effects of various procedures on yeast elimination test procedures (tooth brushing, etc.) were carried out 1 hour after eating the yeast cake. One hour later (2 hrs. after eating the yeast) yeast counts were made in the way just described. The difference between the recovery of the yeast in the control and test counts indicated the extent to which the test procedure had influenced the elimination of the yeast. Ten young adults were used for test subjects. In 8 of these all tests were carried out in triplicate,

[1] Presented at the 21st General Meeting of the International Association for Dental Research, Chicago, March 13–14, 1943. (*J. D. Res.* **22**: 227, 1943). Received for publication November 15, 1943.

in the other 2 individuals duplicate tests were run. Only 1 test was made on a subject in any day.

The principal modification of Knighton's procedure was that a correction factor was worked out to compensate for the different contents of living organisms in the yeast cakes. For this purpose the numbers of yeasts were estimated by making counts on 0.1 gm. of samples taken from 3 different areas on each yeast cake. On the basis of the counts for the various yeast cakes, which showed anything up to a 15 fold difference, the figures obtained from the test counts were proportionated to correspond with an innoculum of uniform size.

The major part of the study, consisting of 265 tests, was carried out during a period of 2 months. The tests included, (a) eating 130 grams of a peeled Sunkist orange, (b) chewing 1 stick of chewing gum (Wrigley's Spearmint) for 30 minutes, (c) eating a Milky Way candy bar, (d) brushing the teeth 3 minutes with a toothbrush and liquid dentifrice (Teel) followed by a rinsing with 150 c.c. of tap water, (e) drinking 110 c.c. orange juice from Sunkist oranges, (f) eating a peach (120 to 180 grams), (g) chewing 2.5 grams of paraffin for 30 minutes, (h) eating an apple (Winesap (60 to 80 gms.) or Gravenstein (160 to 200 gms.)). The following variables should be noted in these tests: Minor variations in time between the tests were unavoidable; considerable variations existed in the numbers of yeasts in the cakes employed (from 30×10^9 to 455×10^9 per gram of yeast); the procedures of toothbrushing were not identical, patients using the brush in their own way; variations occurred in the fruit used, the peaches in 2 lots did not exceed 130 grams but in the third lot they were in excess of 160 grams (these differences showed themselves in proportional differences in the yeast recovered); between the first series of tests and later ones the new season's apples replaced the old season's apples on the market [the former, (Gravensteins) which were also larger in size, proved to be more effective than the latter (Winesaps)].

In addition to the procedures outlined above, a few other tests were made. These included a comparison of the effects of various substances as dentifrices. One hour after eating a yeast cake patients were instructed to use a toothbrush with various "dentifrices" for a period of 3 minutes, followed by a mouth rinse with 150 c.c. of water. Yeast counts were taken in the usual way 1 hour later. The agents used as "dentifrices" were the following: orange juice, (pH less than 3), distilled water, 0.85% sodium chloride, mineral oil, hydrogen peroxide, 2% ammonium hydroxide and a commercial toothpaste. Miscellaneous studies were carried out to determine whether oily materials had any effect in preventing lodgement or causing displacement of yeast. For this purpose subjects rinsed their mouths with 10 c.c. of mineral oil before eating the yeast cake, and ate bread and butter or fried clams as test materials after yeast ingestion. Two tests were carried out to determine the cleansing effect of eating carrot.

Some of the preliminary results suggested that the fairly constant differences in the clearance of yeast from the mouths of the various subjects might be the result of individual variations in the subjects' mouths. To test one such possibility a series of tests was run in which the yeast recovery was compared with the amount of the salivary flow of 21 subjects. This was determined by meas-

uring the saliva collected during 3 minutes chewing of paraffin before the experiment was started (saliva collected for initial yeast count), and at the end of the experiment (saliva collected for final yeast count). Since there seems to be a relationship between the naturally occurring yeast count and caries activity, it also seemed possible that the amount of dental caries or fillings in the mouth might determine the rate of yeast removal in the tests. Therefore, comparisons were made between a group of 8 dental student patients with high caries experience and a group of 8 with a maximum of 2 cavities or fillings.

RESULTS

The initial counts of mouth yeasts made before eating the yeast cake mainly fell between the 500 and 5000 colonies per c.c. Because counts of this size seldom represented as much as 0.1% of those obtained in counts made after the tests, they have no significant bearing on the results, and for that reason have not been taken into account in our calculations. The results of the various yeast counts under control and test conditions for the various subjects varied considerably, some counts being 10 times higher on one day than when the same procedure was followed on another day. Such divergent results have been omitted and our averages calculated only from counts which were of approximately the same order of magnitude. A summary of the results is presented in Table I, which includes both averages for all of the tests and averages which have been corrected by the exclusion of figures derived from test series which showed wide variations. It can be seen that on the basis of the overall averages that the effectiveness of the various agents in removing yeast from the mouth descends in the following order; candy, apple, gum, chewed orange, paraffin, peach, toothbrushing and orange juice. For the selective averages which probably give a more accurate estimate of what happened, results fall in the same order except that apple replaces candy as the most effective agent.

The additional tests gave the following results. The yeast recoveries after mineral oil were greater than those obtained in control counts, the figures being 107%, 640% and 750% of the control recovery. The recovery figures for the tests with clams and bread and butter were 40 and 7.9% respectively. In 2 tests with carrots recovery figures of 2 and 2.8% were obtained.

The results of the tests with various "dentifrices" are given in Table II. In view of the limited number of tests and the variations in the individual results it is impossible to draw any conclusions regarding the effectiveness of the different substances used as dentifrices. Table III shows yeast elimination in relation to salivary flow. The results are indefinite although there is some indication that the yeast was more rapidly removed when there was a relatively high rate of stimulated salivary flow. In the 10 patients with salivary flows of less than 6 c.c. in the 3 minute collection period none gave yeast recoveries of less than 1,000,000 whereas of the 11 with salivary flows greater than 6 c.c. 7 gave counts below this level. The results on the elimination of yeast in relation to dental caries experience are given in Table IV. This table shows that although the initial yeast count in the 8 patients with high caries indices was almost ten times

TABLE I

Average yeast recoveries after test procedures

SUBJECT	CONTROL		CANDY		APPLE		GUM		ORANGE CHEWED		PARAFFIN		PEACH		TOOTH-BRUSH		ORANGE JUICE	
	Cols. × 10^4	% of control	Cols. × 10^4	% of control	Cols. × 10^4	% of control	Cols. × 10^4	% of control	Cols. × 10^4	% of control	Cols. × 10^4	% of control	Cols. × 10^4	% of control	Cols. × 10^4	% of control	Cols. × 10^4	% of control
AP	658	100	30*	4.6	52	7.9	45	6.9	66	10	9*	1.4	88	13	205	31	385	58
AM	1828	100	43*	2.3	70*	3.2	94	5.1	138	7.5	247*	13.7	392	21	649	35	1335	73
LR	1166	100	109*	9.31	133*	11.4	164	14.0	290	24.8	23*	2.0	754	64.4	1162	99.1	955	81.6
ST	1630	100	63	3.9	50*	3.1	36	2.2	65	4.0	46	2.8	162	9.9	188	11.5	590	36.2
EK	1880	100	46	2.4	71	3.8	389	20.7	168	8.9	529	28.2	163	8.7	1365*	72.6	1030	57.4
EL	2357	100	21*	0.9	197	8.4	350*	14	324	13	356	15.1	393	16.7	487	20.6	1193	50.5
TF	242*	100	45	14	19	8	57	23	38	17	51	21	47	20	31	13	153	63
NC	1789*	100	158	8.8	62	3.5	122	6.8	129	7.2	210*	11	355	20	521	29	1356	75
KM	2677*	100	138*	5.2	229	8.6	351	13.1	497*	14.9	396*	14.8	436	16.3	1696	63.4	2235	83.6
EC	2143	100	140	6.5	91	4.25	195	9.1	194	9.1	252*	11.8	562	26.3	786	36.7	380*	17.7
Avs.....		100		5.8		6.2		11.5		11.6		12.2		21.7		41.2		59.6
Selected Averages[1]..				7.1		6.3		11.1		11.3		16.7		21.7		36.7		64.4

* Results based on less than 3 counts or widely varying figures.

[1] Excludes questionable individual averages (*).

TABLE II

Effects of various "dentifrices" on yeast recovery

SUBJECT	CONTROL		ORANGE JUICE		DISTILLED WATER		SODIUM CHLORIDE		MINERAL OIL		HYDROGEN PEROXIDE		AMMONIUM HYDROXIDE		TOOTH PASTE		% OF CONTROL FOR SUBJECT
	Cols. × 10^4	% of control	Cols. × 10^4	% of control	Cols. × 10^4	% of control	Cols. × 10^4	% of control	Cols. × 10^4	% of control	Cols. × 10^4	% of control	Cols. × 10^4	% of control	Cols. × 10^4	% of control	
EK	1880	100	850	45	520	28	1130	60	7100		270	14	2060		460	24	34.2
ST	1630	100	240	15	210	13	290	18	470	29			310	19	330	20	19
NC	1789	100	2700		465	26	1400	78							1000	56	53.4
AP	658	100									170	26					26
"Dentifrice" Avs.....		100		30		22		52		29		20		19		33	67

as high as that in the patients with low caries indices, the final recovery figure, that is the count taken after eating yeast, was no higher in the patients with high caries indices than in those with low caries indices.

TABLE III

Yeast clearance in relation to salivary flow

SUBJECT	SALIVARY FLOW		YEAST RECOVERY	
	No. of Tests	Av. ccs.	No. of Tests	Av. Cols. × 10^4
EL	3	3	4	300
WC	2	4	3	101
EK	1	4	4	161
WR	2	4	2	122
LC	2	4	2	428
JB	2	5	2	270
MD	2	5	2	161
JW	2	5	2	140
EC	3	5	6	225
WH	1	5	1	198
WN	2	6	2	97
SI	2	6	2	59
SG	2	6	2	58
NC	1	6	4	119
DB	1	7	1	37
TF	1	8	4	22
LR	2	9	3	165
TD	1	9	1	23
DA	2	10	2	183
AM	3	12	6	334
AP	1	15	4	84

TABLE IV

Yeast recovery in relation to caries

SUBJECT	INITIAL YEAST COUNT		FINAL YEAST COUNT	
	No.	Av. Cols. × 10^4	No.	Av. Cols. × 10^4
Low caries				
SG	2	34	2	58
JW	2	51	2	140
WR	2	93	2	122
EC	3	14	6	225
SI	2	584	2	59
DA	2	58	2	183
LC	2	117	2	428
MD	2	76	2	161
Average		128		172
High caries				
JB	2	2200	2	270
WC	2	798	2	101
TD	1	1500	1	23
EL	4	319	4	301
WH	1	1600	1	198
WN	2	1060	2	97
DB	2	20	1	37
HN	1	512	1	52
Average.....		1023		135

DISCUSSION

The findings in the major part of this study give definite confirmation of Knighton's conclusions. The study indicates that the toothbrush is not the *sine qua non* of mouth hygiene, and suggests that foodstuffs offer more effective methods. It seems probable that the efficiency of different agents in aiding mouth cleansing is dependent upon several factors, such as (a) the amount of contact or friction with the teeth and mouth tissues, (b) its ability to stimulate salivation, (c) the capacity of the agent to cause yeast or other particles to adhere to it, and (d) the amount of muscular action required in chewing and swallowing. Such an assumption offers an explanation for the efficiency of candy and apple and the relative inefficiency of the toothbrush and orange juice in removing yeast cells from the oral cavity. Candy and apple have frictional capacity as a result of their bulk; they require a relatively large amount of chewing before they can be swallowed; and a high degree of muscular activity is required in swallowing. The candy used was of a particularly sticky type and consequently would have a high adsorptive capacity for the yeast cells. On the other hand the toothbrush and the orange juice had these effects only in a very limited degree. While toothbrushing does offer considerable frictional activity, it is not effective in all parts of the mouth; it does not reach the interproximal surface, nor does it normally exert any friction on the soft tissues.

The counts on the yeast cakes which were made to introduce a correction factor did not offer any significant improvement over Knighton's method. While the counts did reveal quite wide variations in the number of living yeasts in the various lots of yeast cakes, these results did not manifest themselves proportionately in the final results. It seems likely, therefore, that regardless of the number of living yeast cells in a particular cake there is always a sufficient number to saturate the mouth with the test organisms. This conclusion is supported by the consistently high yeast recoveries from some mouths, and the relatively low counts from others, the differences apparently being dependent upon the ability of the particular mouth to retain the organisms.

Our evidence suggests that the amount of saliva secreted might influence this result, there being a somewhat greater removal of yeast where there is a high salivary flow. Our evidence does not, however, indicate that this is the only or even the principal determining factor. It is also interesting to note that the amount of yeast recovered from the mouth was not related to the initial count in the mouth. This can be interpreted as an indication that the initial yeast count is the result of yeast existing in cavities or other stagnation areas in the mouth rather than the result of the yeast content of the whole mouth. Similar tests with lactobacilli might serve to indicate whether their recovery is primarily dependent upon growth in carious cavities or to an increased population in the mouth as a whole.

The results with various "dentifrices" show that the type of cleansing agent used is relatively unimportant. That the reaction of the dentifrice has little effect on its cleansing ability is demonstrated by comparison of the results with orange juice, ammonia and water. A dentifrice containing an abrasive (com-

mercial toothpaste) is shown to have little advantage in the removal of free particles over one with no abrasive. The remarkable constancy of the results in these tests obtained in the mouth of one subject (ST) suggests that the natural cleansing capacity of the mouth is more important than the type of oral hygiene.

The miscellaneous studies support the original thesis that adsorption, salivation, frictional capacity, and muscular activity are the important factors in the removal of particles from the oral cavity. The chewing of bread and butter, for instance, gave results comparable to those obtained by chewing candy and apples. It is interesting to note that on the basis of a very limited number of tests carried out, the most efficient cleansing agent found was carrot, which incidentally required a maximal amount of chewing. That the natural cleansing capacity of the mouth can be interfered with is indicated by the tests with mineral oil. Further tests along these lines might serve to indicate whether any of the commonly used foodstuffs exert the same unfavorable effect.

Perhaps the principal value of these studies on the cleansing effects of foodstuffs and other agents is that they open up an important field for future investigation. Expansion of the present studies using existing methods can well produce much new and important information. Refinement of the basic method is obviously needed and might possibly be approached by using test "meals" of foods containing either biological or chemical tracing agents. If the relative adhesiveness or cleansing effects of common foodstuffs could be worked out, a new approach to oral hygiene and caries prevention might be possible and much needed prophylactic methods, which would be more applicable to the general population than those in use at the present day, might result.

SUMMARY

Tests on the elimination from the mouth of artificially introduced yeast indicate that, in descending order, chewing apples, sticky candy, chewing gum, paraffin and peach were more effective than toothbrushing in cleaning the mouth.

Preliminary tests indicate that carrot and bread and butter greatly aided but that prior use of mineral oil interfered with the cleansing mechanisms of the mouth. Intrinsic factors possibly including the rate of salivary flow seemed to be of more importance in determining the rate of removal of yeast than the nature of the cleansing agents used on a toothbrush.

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FACTORS INFLUENCING TESTS ON THE ABRASION OF DENTIN BY BRUSHING WITH DENTIFRICES[1]

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In previous studies from this laboratory on the abrasion of dentin by brushing with dentifrice materials (1), the procedures were chosen to conform to those conditions which seemed to be present in the toothbrushing practice of the majority of dentifrice users. Toothbrushes, whole human teeth, and a crosswise brushing of the labial and lingual surfaces were employed, and under these conditions wear of exposed cementum or dentin was obtained whenever the tests were carried out with those types of dentifrices which contain abrasives.

There were several details of the procedures which seemed worth further investigation. There might be some question of the influence of the brush itself in such tests, either because of its hardness or the manner in which it was used. It seemed important to ascertain the proper concentration of dentifrice to be employed in making abrasion tests, and the sites at which maximum wear takes place on the test objects. The influence of decalcification upon abrasion resistance was also studied in order to permit proper interpretation of the results of tests on dentifrices.

The literature on abrasion testing has been reviewed in the previous paper except for the publication by Tainter and Epstein (2) which appeared recently. Their procedure makes use of human teeth and toothbrushes, but differs considerably from ours in technical details. To date their procedure has been concerned almost exclusively with enamel[2] and hence a very sensitive method was needed for calipering loss by the test specimens. While the procedure has been used to a limited extent on dentin, it is not adequate for the purpose as will be hereinafter explained.

PROCEDURES

(a) *Crosswise brushing machines.* Whole teeth, toothbrushes and crosswise brushing on the labial or lingual surfaces were used as in the previous method (1), but new machines have been subsequently designed with 4 times as much capacity, and a shadowgraphic procedure has been substituted for the tedious microscopic method of measuring the depth of abrasion.

[1] Presented by title at the 20th General Meeting of the International Association for Dental Research, Chicago, March 14–15, 1942. (*J. D. Res.*, **21**: 335, 1942.) Received for publication November 13, 1942.

[2] Unfortunately this fact was not made clear in a recent publication by these authors (3).

Extracted teeth were preserved in formalin until ready for use, then were cast as previously described in Wood's metal cylinders and returned to water until ready for use. The curved surface of each cylinder was filed flat at 2 spots, one parallel to the labial surface of the tooth and the other at right angles to the first. Notches were ground into the enamel parallel to the direction of brushing on the lingual and labial surfaces. The tooth was then ready for a silhouette to be made of the profile of the labial and lingual sides.

In *fig. 1* a tooth is shown above the lens board of an enlarger in position for a silhouette. By means of a rubber band the flat surfaces and the end of the cylinder were held firmly against the sides of a holder shaped like the inside corner of a box, thus insuring a duplicable position of the tooth with reference to the enlarging lens. The lens board was placed in an enlarger and the silhouette of each side of the tooth, magnified 18 times, was projected onto 9 x 12 cm. film. There were 2 locations along the recorded silhouette that were used as points of reference for measuring dentin wear and hence were protected from the action of the brush. The reference notch in enamel was protected by a copper wire twisted around

Fig. 1. Tooth in position for measuring wear by means of shadowgraph

the tooth, while the dentin adjacent to the Wood's metal was protected by a projection on the clamp holding the tooth. The use of 2 reference points on the side of the tooth on which abrasion is measured, instead of 1 such reference point as in the method previously described by the author (1), has been found to make a small but appreciable reduction in the experimental error of measuring abrasion.

The first model of brushing machine is shown in *fig. 2*. As in the previous method, the boxes were set on an incline and were covered with Ethofoil to minimize evaporation. The special arm shown in *fig. 3*, carried a toothbrush minus the handle, and a vane projecting downward for keeping the slurry of dentifrice stirred. Each of the arms pressed downward with a force of 75 g. on the teeth and moved through a $\frac{3}{8}$-inch stroke at 180 rpm., providing 173,000 strokes in the usual 16-hour period.

After both sides of a tooth had been brushed, the profiles were again shadowgraphed and the maximum depth of wear was measured to the nearest 0.5 mm. by superimposing the reference points on the "Before" and "After" photographs, and measuring the greatest change in the profile. The appearance of such superimposed negatives may be seen in *fig. 4*. To obtain the figure for wear in

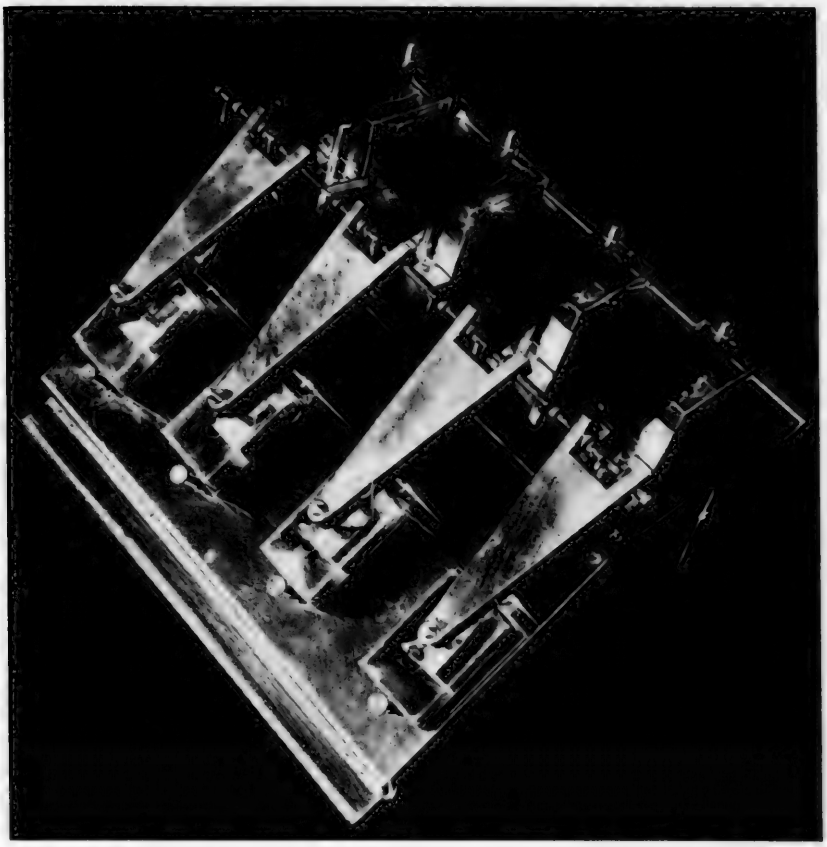

Fig. 2. Machine for testing wear of cementum and dentin by cross-brushing

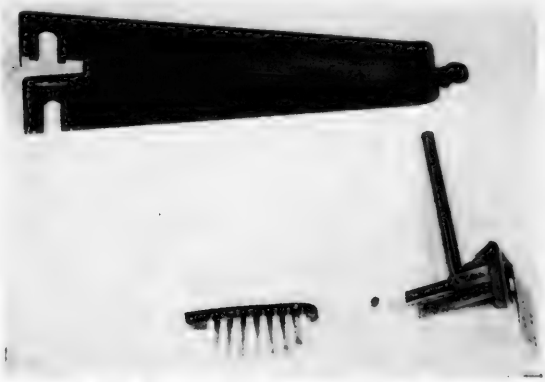

Fig. 3. Special arm used to brush teeth

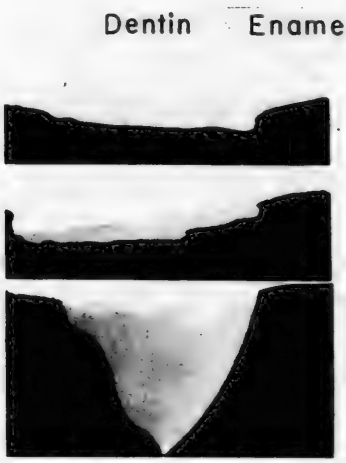

Fig. 4. Examples of method of measuring abrasion. Gray area is part worn away and black area is "after" profile. Dentin at left was protected from wear and enamel at right was resistant to abrasion.

Fig. 5. Second model of cross-brushing machine

terms of microns per 100,000 strokes, the millimeters of profile change were multiplied by 10^8 and divided by the product of the magnification and the number of strokes. When 16 hours of brushing were employed the conversion factor was 32.

Since the measurements by this procedure are affected by such variations among the teeth as hardness, width and shape, the precision cannot be as great as that obtained with other physical tests not depending upon a biological tissue. The standard deviation of a single determination was found to be 50 microns or 35%. With 8 tests the average was calculated to have a standard deviation of 18 microns or 12%, and this was considered to be precise enough for the present purposes.

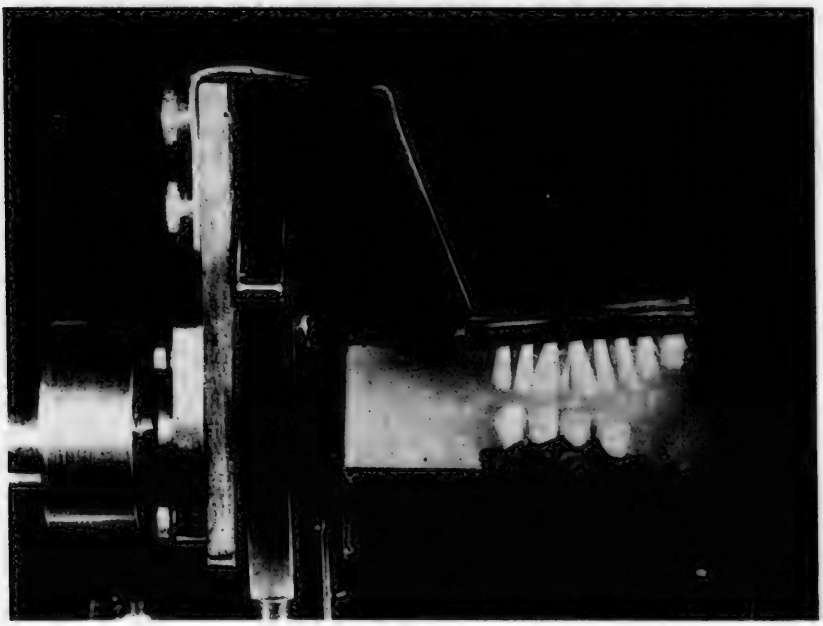

Fig. 6. Machine for testing wear of dentin by occlusalward brushing

A second model of eight-unit brushing machine (*fig. 5*) was designed to provide additional test capacity with greater ease of operation. In principle the machine is similar to the first apparatus, but instead of a load on the brush the force was applied by spring tension. The use of glass containers instead of metal boxes permitted observation of whether sedimentation of the dentrifrice had occurred, and diminished the loss of slurry volume through evaporation. The apparatus is relatively new and has been used only for some of the tests reported in *fig. 8*.

(b) *Occlusalward brushing machine.* The machine used for brushing teeth parallel to their long axis is shown in *fig. 6*. The shaft of a 180 rpm. gear-in-head motor was fitted with a crank pin, $\frac{1}{2}$-inch off center, which was connected with a

4-inch metal rod about $\frac{3}{4}$-inch from the center of the latter. The rod in turn was pivoted at its center point by a bearing parallel to the motor shaft. The bearing was fastened to a block that was free to slide in a vertical position. From the upper end of the rod a clamp for the toothbrush held the brush in position in line with the center bearing of the rod. By this mechanical device it was possible to duplicate rolling, occlusalward brushing, as it is recommend.d by some authorities.

The teeth were mounted in wax in the rectangular box shown in *fig. 6*, and all except 1 or 2 mm. of each root was protected by a base metal casting. Dentifrice was added to the box in a 33% suspension in water. Since the box was supported on a rod parallel to the motor shaft, and kept from tipping only by a spring, each brush stroke rocked the box enough to keep the dentifrice in suspension.

RESULTS AND DISCUSSION

The abrasiveness of the brush. In previous experiments on solid-containing and solid-free dentifrices, no data had been obtained on dry brushes or on brushes moistened with water. There seems to be no published literature indicating an experimental basis for expecting wear by the brush alone, but opinion has been expressed that this is true. (4, 5, 6, 7,8). For this reason tests of the abrasiveness of the brush alone were carried out on the cross brushing machine, and are reported in Table I. The average wear by a dry, medium-hardness, synthetic-bristle toothbrush was found to be 10 microns per 100,000 strokes. Among the individual experiments in Part A there were 5 teeth with no wear and 3 with the minimum measurable notch, 16 microns (0.5 mm. on the negatives). In the brushing experiments with water and in those with unthickened liquid dentifrice the wear was less than with the dry brush, probably because of the lubricating action of the liquid. Likewise the thickening agent in commercial liquid dentifrice probably exerts further lubricating action, for it was found that no wear was produced by brushing with one of these products, either in the present or in the previous experiments (1). In comparison with abrasive-containing dentifrices, the brush itself appears to have negligible abrasive properties toward dentin, since the average wear by leading pastes and powders (1939) amounted to 940 microns, of which a dry brush could account for only 1% of the total and a wet brush for only 0.5%.

Furthermore the hardness of the brush is apparently not a significant factor in influencing the wear by brushing dentin with or without abrasives, judging from Part B of Table I. Tests with liquid dentifrice and 7 brands of hard-bristle brushes, including both synthetic and hog-bristle types, gave no indication of abrasion. In the presence of abrasives the wear of dentin appeared to be similar whether or not the synthetic bristle brushes had been softened with steam prior to the test.

The direction of brushing. In most of the machines described in the literature the brush has moved crosswise over the teeth. This has been a logical first choice in testing, since surveys and personal observation had indicated that

cross-brushing is practiced by the majority of dentifrice users (4, 9, 10). Probably cross-brushing is customary because it is easier than other technics, for it is employed in spite of the facts that other procedures are generally recommended by dentists (4, 5, 11, 12, 13, 14), and that the cross-brushing method has been specifically condemned as likely to be harmful to the gingiva and cervical areas of the teeth (4, 5, 9, 13, 15, 16).

Although there seems to be no experimental basis for the expressed opinion, it has often been believed that an occlusalward method of brushing might avoid abrasion to the cervical portion of the teeth. This question was put to test in the apparatus especially designed for the purpose (*fig. 6*), in which the brush bristles are placed firmly against an artificial gingiva and are moved toward the crown of the teeth by a twist applied to the head of the brush. The first observa-

TABLE I

The relative influence of toothbrushes and dentifrice abrasives in producing wear of dentin

	DEPTH OF NOTCHES IN DENTIN (MICRONS/100,000 STROKES)	
	Individual tests	Average
A. Brush vs. abrasive:		
Dry brush alone	0, 0, 0, 0, 0, 15, 15, 15	10
Brush and water	0, 0, 0, 0, 0, 0, 30	5
Brush and unthickened liquid dentrifice	0, 0, 0, 0, 0, 0, 0, 30	5
Brush and thickened liquid dentrifice	0, 0, 0, 0, 0, 0, 0*	0
Brush and popular toothpastes and toothpowders (1939)	300, 400, 730, 740, 770, 860, 870, 1720, 2070	940
B. Effect of the hardness of the brush:		
Without abrasive:		
Hard brushes (7 brands) and liquid dentifrice	0, 0, 0, 0, 0, 0, 0	0
With $CaHPO_4$ in 33% suspension:		
Hard brushes	110, 190, 270, 450	260
Steam-softened brushes	220, 300	260

* See also experiments reported previously (1).

tions with the machine showed that the brush would not reach the cervical areas if there were insufficient exposure, "festooning" of the artificial gingiva, or severe misalignment of the teeth being tested. On the assumption that the purpose of brushing is to make contact with as many surfaces of the teeth as possible, the conditions were chosen to allow the brush bristles to have opportunity for reaching the cervical area.

Under these conditions 15 teeth were each brushed for 173,000 single strokes, using a 1:2 mixture of a popular toothpowder and water. Three of the teeth were not abraded. The remainder were worn to a greater or less extent, but the appearance of the notches in the dentin differed from those produced *in vitro* by cross-brushing. As may be seen from *fig. 7*, the notches tended to be U-shaped rather than V-shaped, and one was cut diagonally rather than transversely.

Six of the abraded teeth were notched 0–0.5 mm. deep, 4 were 0.5–1.0 mm. and 2 were 1.0–1.5 mm. in depth.

It seems evident that abrasion of cementum and dentin will be obtained when teeth are brushed longitudinally. However, when the longitudinal and transverse brushing figures are made comparable by calculating each to the wear per meter of contact with the brush, there is about 2 to 3 times as much wear with the cross-brushing. Such distinction might be expected from the tendency which the bristles possess for concentrating in the notch during cross-brushing, and diverging out of the notch during longitudinal brushing.

The site of maximum wear on a test object. The difference between the wear obtained by cross-brushing and by longitudinal brushing of teeth raised the question of how much the shape of a test object influences the abrasion that it receives. Early in a test on abrasion by cross-brushing, the profile of the groove is U-shaped and as the wear proceeds the grove becomes progressively more wedge-shaped. There seems to be an explanation for this in the observation

Fig. 7. Examples of cervical notches produced by occlusalward brushing

that brushing with even slight pressure forces the brush bristles to slide into the depressions of a surface being brushed, causing most of the frictional force to be applied at these sites which have already received the most wear. It seemed of interest to evaluate this tendency under conditions which made the shape of the test object the only variable.

Ivory was chosen for these tests because it afforded a better source of dentin than human teeth for test pieces of convenient size and the desired uniformity. After cylinders of the material were turned on a lathe, grooves of varying size and depth were turned into the surface of the cylinders. When one end of the cylinder had been cut to fit the clamp on the machine, the specimen was fixed in place and brushed parallel to the direction of the grooves with a slurry of dentifrice grade $CaHPO_4$. Silhouettes on film were prepared before and after brushing according to the technics already described. Tracings of the before and after silhouettes are reproduced in *fig. 8* along with a sketch of the cross-section of the brush drawn to the same scale. The brush movement, of course, was in a direction perpendicular to the plane of this figure. Specimens E and F were brushed

86,000 strokes and the remainder, 172,000 strokes. The site of wear on the ivory is indicated by the hatched lines sloping to the right.

In every case the abrasion took place almost exclusively in the notches rather than on the crests. On the average there was about 9 times as much wear in the depressions as on the crests. The sharp groove on specimen B brought the brush bristles together as shown by the dotted lines, while the crests in C and E caused a divergence of the bristles around the peak, resulting in wear on either side but very little on the crest itself.

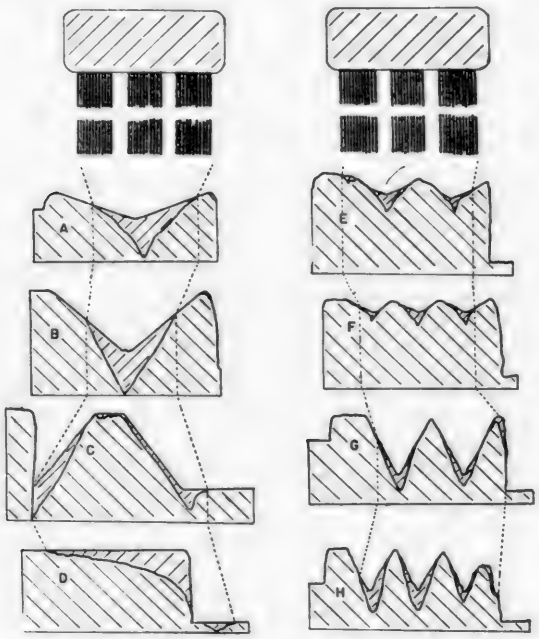

Fig. 8. Site of abrasion when notched ivory test objects are brushed with abrasive. Wear (shown by diagonal shading) occurs almost exclusively in grooves rather than on crests.

In other words, *the formation of V-shaped notches is a fundamental characteristic of abrasion of dentin by brushing with abrasives*, following as a corollary from the finding that abrasion is most rapid at the bottom of a notch. The process may be compared with the mechanical wear of a hillside by water, resulting in gullies which progressively deepen and thereafter account for the main loss of soil. In studying the soil erosion of a hillside, observations on the depths of the gullies are more important in indicating the extent of soil loss than measurement of the surrounding elevated areas. Likewise, in studying dentin abrasion by brushing with abrasives, the wear of the depressions deserves more attention than the wear of the crests. This is why the method of Tainter and Epstein (2), which limits observation to the crests of the test object, is of quite restricted

usefulness in the study of abrasion of dentin. It is uncertain to what extent the same objection might apply to the method when it is used on enamel.

The dilution of dentifrice. In the previous study on abrasion, 2 parts of water were used to dilute dentifrices prior to the tests. The selection was based on relatively few experiments, and another investigator chose a greater dilution (17). Hence it seemed important to investigate the question further, and if the proper dilution were not 2 parts of liquid, then it would be necessary to ascertain how much error was being introduced into the tests.

Information was needed on how much dilution of dentifrice occurs on the toothbrush during the customary brushing of the teeth. This was determined by presenting each of several subjects with a tared brush. He was requested to brush his teeth according to his usual habit. After the interval specified the

TABLE II

The dilution of dentifrice on the brush during the usual toothbrushing practice

DENTIFRICE	DURATION OF BRUSHING	INDIVIDUAL VALUES	AVERAGE ABRASIVE CONCENTRATION	DILUTION (PARTS OF LIQUID ADDED)
Toothpaste A	Control	31, 42, 42, 44, 45, 47	44	—
	0 sec.	28, 31, 37, 41, 43, 49	38	0.2
	10 "	10, 14, 20, 21, 22, 23	18	1.4
	30 "	11, 13, 19, 25, 26, 29	25	0.8
	60 "	6, 6, 6, 7, 11, 19	9	3.9
Toothpowder B	Control	81, 86, 94, 97, 98, 100	91	—
	0 sec.	44, 57, 61, 70, 72, 76	63	0.5
	10 "	7, 9, 19, 28, 28, 32	21	3.3
	30 "	11, 12, 16, 19, 20, 23	17	4.4
Toothpowder D	Control	62, 65, 66, 69, 69, 70	67	—
	0 sec.	17, 25, 26, 28, 32, 33	27	1.5
	10 "	9, 10, 10, 11, 11, 20	12	4.6
	30 "	1, 2, 5, 10, 12, 12	7	8.6

brush was recovered and re-weighed to determine the weight of diluted dentifrice adhering, and the weight of abrasive was determined by filtration and drying. By comparison with controls on the same dentifrices, the average dilution of dentifrice was calculated.

The results on dentifrice dilution are given in Table II. Part of the subjects wet their brushes before applying the toothpaste A, and this on the average diluted the dentifrice by 0.2 parts (by weight) of liquid before the brushes were placed in their mouths. Thirty seconds after the brushing had begun, saliva had increased the dilution to 1.4 parts, and by the end of the brushing period the dilution had become 3.9 parts. Thus the range of dilution for this dentifrice was 0.2 to 3.9 parts of water, and a dilution of 2 parts falls in about the middle of this range.

With toothpowders, however, the range is so great that it is difficult to ascertain the proper dilution to be chosen. The brushings with powders began at dilutions of 0.5–1.5 parts of liquid, rose to 3.3–4.6 parts in 10 seconds, and were at 4.4–8.6 parts in 30 seconds. When the abrasive concentrations are considered, it appears that a toothpowder is at first more abrasive than a paste prepared from the same solid, but within 30 seconds it becomes less abrasive than the paste. This means that any dilution that is chosen for testing powders will be arbitrary, but of the logical choices probably 2 parts is as satisfactory as any other.

As a test of whether a single dilution approximates the extent of wear produced by conditions more closely approaching those in the mouth, comparisons were made between the abrasion with a 33% slurry of the dentifrice, and that with the same dentifrice applied intermittently to the brush. The data in Table III show the abrasion by 2 popular dentifrices when these were applied by hand every 14 strokes for four hours. Although intermittent application produced less wear with the powder and more wear with the paste, the results are of the same order of magnitude.

TABLE III

Influence of the method of applying the dentifrice to the brush

	DENTIN ABRASION (MICRONS/100,000 STROKES)	
	Powder A	Paste A
33 percent slurry kept in contact with the brush	1720 1300	730*
Undiluted dentifrices applied intermittently to the brush	1330 1080	1080 1560

* 1939 samples of dentifrice.

A dilution of a dentifrice does not usually bring about a proportionate reduction in abrasiveness. In a series of tests on a dentifrice grade dicalcium phosphate (Table IV), an 80% decrease in concentration just halved the abrasiveness of the slurry. If the same relation holds for the dentifrices tested in the dilution tests, the abrasiveness of Toothpaste A is half as great at the end as at the beginning of the brushing, and the abrasiveness of the toothpowders falls only 60% during the first 30 seconds of brushing. By the same reasoning, it was calculated that the unavoidable evaporation during overnight abrasion tests with the apparatus in *fig. 2* does not affect the abrasion values more than the error of measurement, although the abrasive slurry becomes 15% more concentrated on the average. Smith (18) and Epstein and Tainter (3) also noted that small changes in the concentration of an abrasive do not have any noticeable effect on its abrasiveness.

Probably the rate of abrasion is independent of concentration as long as the slurry is fairly thick, because of the fact that rubbing surfaces are thoroughly covered by abrasive and a change of concentration merely alters the thickness

of the abrasive layer between the surfaces. However, as the slurry becomes more dilute there is probably some critical concentration at which the layer of abrasive becomes too thin to carry the whole load, and part of the brush bristles begin to make contact with the tooth surface. As already mentioned, the brush bristles alone do not produce appreciable wear. At the critical concentration, then, the abrasion of dentin will probably decrease very rapidly as the abrasive concentration is further lowered.

The influence of decalcification on the abrasion resistance of dentin. In routine tests on the abrasiveness of dentifrice solids, only sound dentin would be used for test objects. However, decalcification of dentin occurs sometimes in the mouth, and tests on dentin affected by decalcification would be of interest, especially since decalcification of dentin has sometimes been considered as a cause or a contributory factor in the formation of cervical notches *in vivo* (15, 19, 20, 21, 22, 23, 24, 25).

TABLE IV

Change of abrasiveness with concentration of the abrasive

	PERCENT OF DENTIFRICE GRADE $CaHPO_4$				
	6.6	9.0	14	20	33
Abrasion of dentin, microns per 100,000 strokes	260 320 580 805	290 465 580 740	180 510 530 700 800 900 910	820 920	780 930 1020 1120
Averages.....	490	520	650	870	960

In order to prepare decalcified cervical dentin, 8 extracted teeth were covered with paraffin except for small cervical areas on the labial and lingual sides, then immersed in 5% lactic acid for 70 hours. After removal of the paraffin the teeth were shadowgraphed and x-rayed, and from the x-rays it was determined that decalcification of cervical dentin was essentially complete. The labial surfaces of the 8 teeth were brushed first at the site of decalcification for a 16-hour period with water, next for the same length of time with a liquid dentifrice, and finally with various leading pastes and powders. The extent of abrasion that resulted is presented in Table V, along with the corresponding data on sound dentin for comparison.

Decalcification seems to have raised the dentin's susceptibility to abrasion by the moistened brush, and made it possible for a trace of wear to take place when the affected tissue was brushed with a liquid dentifrice. This would be anticipated since the tissue had become soft enough to be torn with a probe. It is surprising, however, that the wear on brushing with 6 leading pastes and powders appears to be less on decalcified than on normal dentin. If the figures

on Paste A are omitted from the average because the abrasion values of different samples of that brand are extremely variable, the decalcified and sound dentin averages merely become equal.

These findings seem to show that decalcification is not a factor of any significance in producing cervical notches in dentin. This agrees with Miller's observations (26). The extent of wear of acid-softened dentin by the moistened brush is about one-fifteenth of that which was produced by abrasives on acid softened dentin of the same teeth.

TABLE V

A comparison of the resistance of sound and decalcified dentin to abrasion

MATERIAL USED WITH BRUSH	ABRASION (MICRONS/100,000 STROKES)			
	Decalcified dentin	No. tests	Sound dentin	No. tests
Water	50	8	5	7
Liquid dentifrice	15	8	0	15
Toothpastes and powders:				
Powder A	490	1	400	4
" B	1050	2	2120	4
" C	1820	1	920	4
Paste A	170	2	810	4
" B	530	1	550	4
" C	560	1	400	4
Average	770		870	

SUMMARY

A procedure is described for testing the abrasiveness of dentifrices by means of machines capable of brushing 8 teeth simultaneously, and a shadowgraphic method for measuring cervical dentin wear. Other equipment was devised for determining whether abrasion takes place on exposed dentin when it is brushed in an occlusalward direction.

(1) The brush itself was found to have insignificant abrasive power, since a dry brush abraded dentin only 1% as much as the average of leading pastes and powders. The wet brush was less abrasive.

(2) When teeth with exposed cementum were brushed in a machine in a direction parallel to their long axis, wear of cementum and dentin was produced on a majority of the teeth, but the average rate of abrasion was less than that found with cross-brushing.

(3) When notched ivory cylinders were brushed with abrasive, little or no wear occurred on the crests of the surfaces brushed, and the maximum abrasion developed in the deepest depressions.

(4) Studies were made to determine the proper concentration of dentifrice for use in testing abrasion. It was found that dentifrices are rapidly diluted during toothbrushing, and a 33% concentration of dentifrice is within the range of dilutions observed *in vivo*.

(5) Decalcified dentin exhibited about the same resistance to wear by popular abrasive-containing dentifrices as did normal dentin. Although the resistance to wear by a moist brush was decreased, decalcified dentin was worn by the moist brush merely about 7% as much as by solid-containing dentifrices.

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STUDIES OF THE EFFECT OF INGESTION OF CITRUS FRUIT UPON GINGIVAL HEMORRHAGE[1]

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Although the oral cavity constantly harbors bacteria of various species the problem of reducing the number of the pathogenic organisms is difficult. Efforts have been directed toward reducing the numbers of bacteria in the mouth or toward diminishing the pathogenic effects of the oral flora. Agents of the bacteriocidal type are more or less irritating and it is necessary to depend on some mechanism other than direct killing of the organisms. The oral environment is as important as the actual bacteria in most mouth lesions. The general opinion is that complete disinfection of the oral cavity is impossible.

Studies on the effects of various dentifrices and methods of oral prophylactic treatment have demonstrated the impractibility of sterilizing the oral cavity (2). Gardner (3), investigating conditions following extraction of teeth, found that attempts to sterilize the field of operation were undesirable unless the sterilizing agents themselves did not provoke inflammatory reactions. He found socket medication inadvisable because irritation, not sterilization resulted. Appleton (4) noted that the use of a non-medicated dentifrice and mouth wash may reduce the number of bacteria by about 40% for a short period of time. Leonard and Feirer (5), from study of 41 different commercial dentifrices, found that none of the tested agents produced any significant reduction in the bacterial content of mouth washings. Howith and Fleming (6) and Hine and Bibby (7) observed wide differences in the oral flora from time to time and from one mouth area to another within the same individual. Mead (8) showed that sodium ricinoleate, while devoid of bactericidal qualities as indicated by its phenol coefficient, reduced the bacterial count when used as a mouth wash, and maintained the low count for considerable time. This drug was contrasted with hexylresorcinol which has a high phenol coefficient but which was less effective in the mouth.

Bleeding from the gingivae is associated with various stages and types of periodontal disease. Bleeding gingivae should not be overlooked as possible portals for entry of infection. Okell and Elliott (9) have observed that bacteria may be found in the blood stream following extraction of teeth with periodontal disease. Any of a number of factors may initiate these periodontal lesions. Black (10) directed attention to the role, in periodontitis, of faulty restorations with improper contour, poor contact points and overhanging margins allowing food impaction or directly impinging on the soft tissue. He also incriminated

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improperly constructed complete and partial dentures. Foreign bodies, such as tooth brush bristles, and also occlusal trauma are well recognized as etiologic agents in periodontoclasia. Gottlieb (11) suggested the term "Schmutz pyorrhea" for that type of periodontitis resulting from accumulation of debris and calculus at the gingival margin. He believed that about 90% of periodontal disease had this cause. Ziskin (12) reported periodontal lesions associated with endocrine disturbances and showed the results of hormonal therapy.

As early as 1772, Lind (13) observed improvement in the gingiva in scorbutic patients following the use of a diet containing fresh fruits. Szent-Gyorgyi (14) prepared hexuronic acid (synthetic ascorbic acid) which is now known as cevitamic acid. Wolbach and Howe (15), using orange juice as a source of ascorbic acid showed that vitamin C is essential to proper healing of wounds, the fibroblasts failing to form collagen and the endothelial cells not producing functioning capillaries in its absence. It was then possible to explain why the alveolae rarefied and the teeth loosened as a cardinal symptom of ascorbic acid deficiency. Boyle (16) pointed out the similarities between ascorbic acid deficiency disease in the mouths of guinea pigs and the so-called systemic or diffuse atrophy type of periodontoclasia in man, and that the breakdown of the gingival attachments in this type of periodontal disease is a late complication. He also called attention to the fact that the therapeutic dose of ascorbic acid, for individuals long an inadequate diets, may be many times the maintenance dose. Hanke (17) stated that gingivitis is associated with bacterial invasion which can occur only when the tissue resistance is lowered. He also stated that bacteria, plaques and calculus might be present but that inflammation of the gingivae may not occur in individuals consuming much citrus fruit, and that treatment by change of diet to include much citrus fruit, alone, will cure gingivitis. Hirshfeld (18) stated that gingival symptoms are occasionally the most prominent phenomena of scurvy with marked gingival color change from pink to rose to purple to dark blue being noted. Zilva (19) also observed the teeth among the first tissues affected by scurvy. Ochnell (20), in 22 cases, noted gingivitis as the most prominent symptom.

VITAMINS C AND P

Hemorrhagic disturbances have been reported successfully treated by administration of citrus fruits and these results have been attributed to vitamin C. Scurvy in the mouth is characterized by gingival hypertrophy, inflammation, congestion and hemorrhage with dark, bluish-red discoloration of the tissue. The treatment that is generally recognized is administration of lemon juice, orange juice, grapefruit juice, tomato juice, cabbage juice or other vitamin C rich foods. The reduction in hemorrhage in scorbutic gingivae has been presumed to result from the vitamin C administered. It is significant, however, that in most studies of caries and periodontosis the citrus fruit rather than synthetic ascorbic acid was used. Szent-Gyorgyi (21) reported the presence in extracts of Hungarian red pepper, and later in lemon juice, of a substance other than ascorbic acid which would control certain types of hemorrhagic disturbances,

i.e., thrombocytopenic purpura, diabetes mellitus, certain infectious disease, etc. Szent-Gyorgyi and his associates identified the substance which restored capillary walls to their normal states of permeability as vitamin P (permeability vitamin) and found hesperidin as the active substance. This same investigator observed that experimental scurvy was due to deficiency of vitamins C and P, not vitamin C alone, but that failure to produce demonstrable, distinct physical signs delayed discovery of vitamin P. Vitamin P deficiency, however, when accompanying avitaminosis C modified the symptoms of ascorbic acid avitaminosis and, scurvy produced by deficiency of vitamin C in the presence of abundant vitamin P, differs from ordinary scurvy. The symptoms of the purely ascorbic acid deficiency scurvy in rabbits are recession of the gingivae and swelling of the joints with death in 6 to 8 weeks. It may be concluded, according to Szent-Gyorgyi, hemorrhage of ordinary scurvy is caused by vitamin P deficiency and that the more rapid emaciation and death in animals is due to combined vitamin C-vitamin P deficiency.

Knowledge of vitamin P (hesperidin) is increasing but a survey of the literature suggests that little is known in a definite sense. The possible relationship between vitamin P and vitamin K are of interest. Vitamin P administration will return capillary resistance to normal in certain diseases but is without effect in hemorrhage due to arteriosclerosis, aneurism, etc. Scarborough and Steward (22) reduced petechial hemorrhages, produced by pressure and occurring spontaneously after administration of arsenic or bismuth, by oral administration of hesperidin. Jersild (23) used vitamin P to treat, successfuly, a case of Schonlein-Henoch's purpura that failed the respond to ascorbic acid therapy. Lojas (24) claimed vitamin P to be of prophylactic value, clinically. Moll and others (25) have disagreed with Szent-Gyorgyi in regard to the presence of vitamin P. Vitamin P (citrin, citris flavon) has been obtained in crystalline form, 2 grams being extracted from 70 liters of lemon juice.

EXPERIMENTAL

The influence on the microbial flora of the mouth when citrus fruit was added to an otherwise normal diet was investigated. Two groups of individuals were utilized: one group fed fresh grapefruit in addition to the daily diet and the other group receiving no special allotment of citrus fruit. Inmates of the District of Columbia Penal Farm, Lorton, Va., were used for the study because of convenience and because of the possibility of controlled diet. The men were available at any time for examination, the men selected for study had been on the regular prison diet for at least one year, eliminating effects of recent dietary changes, and would remain under term for duration of the study. Cooperation was voluntary.

Data were obtained on 131 men, 67 in the test group and 64 in the control group. The ages ranged from 19 to 55 years, white and negro men being included. Both groups received the regular prison rations, and in addition, $\frac{1}{2}$ grapefruit was served to each of the 67 test group individuals with each meal, i.e., 3 times per day. All fruit used was grown in Florida and purchased on the

open market. Grapefruit was first added to the diets during the second week in January, when common colds and other respiratory infections were on the increase.

Examinations of the oral microbial flora were made on saliva collected periodically from each individual. The flow of saliva was stimulated by chewing sterile paraffin. Initial examinations were made prior to the use of grapefruit, and monthly for 6 months. Bacterial counts were determined in the following media: (1) plain nutrient agar, to determine total numbers of the general saprophytic types, (2) neopeptone tomato juice agar (pH 6.6), for aciduric organisms, (3) blood agar, for those organisms requiring animal protein for growth. Veal infusion agar (pH 7.4) was used as the blood agar base, 5 per cent sterile defibrinated horse blood being added aseptically to the medium just before use. Nutrient agar plates were incubated for 48 hours at 37°C.; tomato juice and blood agar plates were placed in an atmosphere of 10 per cent CO_2 and incubated for 5 days at 37°C.

RESULTS

The average initial bacterial numbers per cc. of saliva among the test group were: Plain nutrient agar 48,238,000; tomato juice agar 33,780,000; and blood agar 104,655,000. After the ingestion of grapefruit for 3 months 81% of these individuals maintained decreased counts, whereas only 39% of the control group showed a continuous decrease. The percentage decrease was not as great for the test group after the fourth and fifth months, possibly due in part to the fact that the grapefruit was not being fully consumed regularly by all men.

Initial examination showed low blood levels for vitamin C, 140 men having an average of 0.45 mg. per 100 ml. of plasma. The optimal level is considered to be about 1.0 mg. per 100 ml. The ascorbic acid levels of the blood of these receiving grapefruit supplements rose to a maximum average value of 1.1 mg. per 100 ml. after 3 months. The introduction of fresh vegetables in the diet during these months was apparently of no effect in so far as vitamin C levels were concerned. An increase in vitamin C intake during the warmer months is apparently essential for maintenance of blood ascorbic acid concentrations as observed by Belser, Hauck and Storvick (28).

The average ascorbic acid level in the blood as determined before grapefruit feeding for 140 men was 0.45 mg. per 100 ml. of plasma. Of the 67 individuals fed grapefruit 22 reach a concentration of more than 1.4 mg. per 100 ml. during 5 months of feeding. The highest value was 2 mg. per 100 ml. During the first month 1 subject exceeded the 1.45 mg. level, during the second month 5 more, during the third month 9 more, 5 more during the fourth month and 2 more in the fifth month. In another group of 8 men the ascorbic acid level never was observed to exceed 1.0 mg. per 100 ml. during the time of feeding. The remaining 40 individuals the maximum levels varied between 1.0 and 1.4 with an average 1.1 for the entire group after 3 months. The wide variation in the blood level was not due to losses in the urine for low ascorbic acid blood levels

were accompanied by low urine levels although each individual had approximately the same intake. The ascorbic acid levels of both groups declined somewhat during the last 2 months of the study, May and June, when the temperature rose and weather was uncomfortably warm.

The average ascorbic acid content of the urine in the group of 140 men was initially 0.6 mg. per 100 ml. A marked increase in this figure was observed, in the group eating grapefruit, between the 11th and 23rd days and thereafter remained at 4 to 6 mg. per 100 ml.

TABLE I

Results of administration of grapefruit to 70 men, compared with 65 men receiving the same rations without supplemental citrus fruit

	TEST GROUP	CONTROLS
Ascorbic acid blood level (mg/100 ml)		
At beginning (average)	0.46	0.43
After 1 month (average)	0.84	0.41
After 2 months (average)	1.03	0.43
After 3 months (average)	1.15	0.42
After 4 months (average)	1.03	0.39
After 5 months (average)	0.95	0.37
Ascorbic acid urine level (mg/100 ml)		
At beginning (average)	0.60	0.60
After 3 months (average)	6.1	0.90
After 5 months (average)	4.2	0.70
Bleeding time		
At beginning (average)	2.3	2.0*
After 5 months (average)	2.5	2.4*
Clotting time		
At beginning (average)	4.5	4.5*
At end (average)	4.6	5.0*
Gingival condition		
Periodontal disease at beginning	90%	62%
Showing marked improvement in 5 months	91%	0%
Showing slight improvement in 5 months	8%	2%

* 20 men only.

Physical examinations made at regular intervals showed, for the test group, increase in appetite, improvement in bowel elimination, feeling of well-being and improved general health.

Dental examination of the test group showed improvement in tone of the oral tissues, and decrease in gingival bleeding among a number of men who had had severely bleeding gingivae. No decalcification or other deleterious effect on the tooth enamel was noted.

Bleeding time and coagulation time were determined for all the test group and for 20 men in the control group prior to the feeding, mid-way in the feeding and at the completion of the study. Those in the test group showed an average of 2.3 minutes for bleeding time and 4.5 minutes for coagulation time at the beginning, 2.5 minutes and 4.0 minutes after 2½ months and 2.5 minutes and 4.6 minutes at the end of the study. The figures for the 20 controls were 2.5 minutes and 4.5 minutes at the beginning, 2.6 minutes and 4.6 minutes after 2½ months and 2.4 minutes and 5 minutes after 5 months.

DISCUSSION

The individuals receiving supplementary citrus fruit showed increase in the ascorbic acid blood level. Those that failed to increase their blood levels to the optimal did not excrete it in their urine as indicated by their urine ascorbic acid studies. There is not complete agreement among authorities as to the optimal vitamin C concentration. Ingalls (26) gives the following figures: 1–2 mg. per 100 ml. of plasma, saturation; 0.7–1.0 mg. per 100 ml., normal; 0.5–0.7 mg. per 100 ml., low normal; 0.3–0.5 mg. per 100 ml., suboptimal; 0.15–0.3 mg. per 100 ml., asymptomatic; below 0.15 mg. per 100 ml., scurvy. On this basis the individuals observed before feeding had suboptimal levels. Of the 70 fed grapefruit 62 reached a level considered as "saturation" by Ingalls, while 8 never reached above the "normal" level. The fact that the urinary ascorbic acid was not excessive for the latter 8 suggests that they utilized their vitamin C in some manner.

The statistical data suggest a remarkable effect of ingestion of grapefruit on bleeding gingivae, soreness and tenderness of the gingivae and allied symptoms. The men were well satisfied with the results and said that their mouths and general feeling were improved. The bleeding and coagulation times were normal for the men before and after ingestion of the extra citrus fruit.

It is apparent that some dietary factor or factors was supplied by the ingestion of 1½ grapefruit per day. It is not shown in this particular experiment whether the decrease in the number of bacteria resulted through local or systemic factors. Gies (27), cited King as maintaining that acid substances would inhibit the growth of bacteria and he also credited Black with the statement that certain acids placed in the mouth inhibit bacterial action. Knighton (29) has demonstrated detergent action of certain foods which must also be considered a factor.[2]

SUMMARY

Seventy men were fed 1½ grapefruit per day in addition to the regular rations for 5 months. A group of 65 men on the same rations without added citrus fruit served as controls. Increased ascorbic acid blood levels, increased ascorbic acid urine levels, improvement in gingival condition and in general health were observed. No deleterious effect on the enamel was noted.

[2] See also the paper by Turesky and Bibby, *J. D. Res.* **23**: 51, 1944 (this issue). (Ed.)

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